STUDIES ON PLAIN MUSCLE

by

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The experimental study of the action of plain muscle tissue presents certain difficulties which one does not usually encounter in examining skeletal muscle. While in the case of plain muscle it is a simple matter to record accurately the slow movements that it undergoes, in comparison with the rapid twitches seen in skeletal muscle, yet these records, as usually obtained, have little meaning so far as the individual muscle fibres are concerned. The twitch-curve of a parallel-fibred skeletal muscle, like the frog's sartorius, is an accurate representation of the twitch-curve of a single muscle fibre; but the corresponding curves for a piece of plain muscle, in the way in which they are usually obtained, even assuming that all nerve ganglia have been removed, do not represent the behaviour of a single muscle fibre, but rather the effect of the contraction of such a fibre in a very viscous tissue, and one which conducts impulses relatively slowly. In order to get a clear understanding of plain muscle tissue it is necessary to have, in the first place, a more accurate knowledge of its intimate structure; it is useless to attempt to understand the action of such a tissue when essential facts, such as the nature of its innervation, the presence of cell membranes, the functional existence of myofibrils, etc., are not known.

In this paper I shall, therefore, give an account of the structure of plain muscle tissue, so far as the microscope is able to reveal it, and, taking partly the facts of skeletal muscle as an analogy, shall try to show that at any rate the more obvious features of plain muscle activity can be given a fairly simple interpretation.

The Structure of Plain Muscle Tissue.

A piece of plain muscle tissue, such as one of the muscular coats of the intestine, or a retractor muscle such as occurs especially commonly in the lower animals, consists of numerous short spindle-shaped cells, each about ·2-·3 mm. in length (24), firmly held together by branching connective tissue cells, and separated from one another by an intercellular fluid.

A. *Structure of the Muscle Fibres.* Great difference of opinion exists among histologists at present concerning the organization of plain muscle fibres. While most observers consider the myofibrillae as having a real existence in the living cell, others take the view that they are formed during the process of killing the cells by the action of the fixing reagents. The existence of a cell membrane, while it is affirmed by some observers, is denied by others; by some, indeed, plain muscle tissue is looked upon as a great syncytial mass, none of the cells having definite cell membranes, and all being connected by "protoplasmic bridges" that bring one fibre into direct communication with others. The nature of the nerve terminations is not accurately known; and the granules which surround the nucleus have received no functional interpretation, though some workers appear to regard them as undifferentiated cell protoplasm.

The observations here recorded have been made mainly on the large plain muscle fibres of the frog's stomach, and especially on the fibres from the pregnant mammalian uterus. Here, as is well known, the fibres hypertrophy enormously during the progress of pregnancy, and present, towards full term, a very suitable object for examining the minute structure of plain muscle cells. The preparations have usually been fixed with Bouin's fluid, and stained with iron haematoxylin.

The cell membrane can, under these circumstances, be seen to be quite a definite membrane covering the muscle cell. In the cells from the gravid uterus, in fact, it is moderately thick, certainly thicker than the fibrils, and of its existence there can be no doubt whatever. It was here that Kölliker saw it especially clearly.

Protoplasmic connecting bridges, on the other hand, have no real existence. The marked development of connecting pieces in cardiac muscle has doubtless lent support to the view that similar structures might exist in plain muscle also, but the most careful examination that I have made of the fibres has failed to reveal their presence. Schultz (12) has even described a definite organization of these connecting bridges, regarding them as composed, in part at any rate, of the myofibrils. The figures that he gives, however, might be interpreted equally readily as representing coagulated intercellular fluid. Definite protoplasmic connecting bridges have also been seen by M. Lewis (14) in plain muscle fibres in tissue cultures. The cells that were examined in this case, however, were embryonic myoblasts, very similar in appearance to embryonic cardiac muscle. They showed only a few myofibrillae after fixation, and were obviously quite different even in external shape from fully differentiated plain muscle as it occurs in adult animals. The best argument for the non-existence of such bridges is that given by Grützner: he found that when the frog's stomach was in a state of tonic contraction, during starvation, the walls were at least twenty contracted muscle

fibres in thickness. When the stomach was relaxed and distended with food the wall was only two or three fibres in thickness. A very marked "sliding" of the fibres over one another had therefore occurred, and this could not take place if protoplasmic bridges were present.

There can be no doubt, on the other hand, that the myofibrillae, which can be seen in well-made preparations running throughout the length of the fibres in great numbers, do really occur as such in the living cell. It is true that often, especially in living cells, they are not visible (14, 19), but this does not prove that they do not exist. It merely means that their refractivity does not differ enough from that of the interfibrillar fluid which surrounds them to render them recognizable. It could also be pointed out that so definite a structure as the infusorian nucleus is often a most difficult object to detect in the living organism.

The proof that the myofibrillae are really morphological entities within the living cells, although not visible there, is twofold. When one examines the greatly hypertrophied plain muscle cells of the pregnant mammalian uterus, these fibrils are seen to be considerably larger than usual. Increase in the bulk of the cell, just as in the cells of growing insect larvae (27), is not accompanied by further cell differentiation; if, therefore, the myofibrils were merely the result of a coagulation process produced by the action of the fixing reagent on a homogenous mass of protoplasm, as some writers believe, then one would not expect an increase in the *size* of these fibrils as hypertrophy proceeded. A further proof that these fibrils are present as such in the living cell, and, moreover, that they are concerned in the process of contraction, is that when we examine them in regions where shortening has occurred, they are seen to be considerably thicker than elsewhere.

For these reasons, therefore, it must be concluded that the myofibrils of plain muscle do occur as such in the living cells, and that they are functional in the process of contraction, just as is the case in skeletal and cardiac muscle. Partly by microscopic examination, and partly from analogy with skeletal muscle, a definite structure may be attributed to these fibrils. When one examines, under very high magnification, the fibrils in the hypertrophied mammalian uterus, *these can very frequently be seen to possess, even in thoroughly relaxed muscle, an ill-defined transverse striation*, just as occurs in skeletal muscle, though not so pronounced. I have seen it also in the fibrils of the adductor muscle of the bivalve *Mytilus*. A number of such fibrils are shown in Figure 3 (2). The striation is never very distinct, and often cannot be seen at all; it should be remembered, however, that even in cardiac muscle the striations of the individual fibrils are also usually very difficult, sometimes impossible, to see, and that it is only through the fact that the two excitable membranes of Krause impose a more marked organization upon the fibres, the sarcomeres being arranged very regularly side by side, that it becomes possible to see "striations" in the fibres as a whole. For

these reasons, I believe that, just as in skeletal and cardiac muscle, so in plain muscles the fibrils possess striations; much more difficult to see, however, since the fibrils are ever so much more delicate, and because they are not regularly arranged in "striation" so far as the entire fibre is concerned. Moreover, various observers—Engelmann, Schultz (24), and others—have observed double refraction towards polarized light in plain muscle. In skeletal muscle this is a property of the "spongy network" in the middle of each sarcomere. Although the extreme minuteness of the fibrils does not enable us directly to observe the structure of these fibrils more closely, yet it seems to me not unjustifiable to conclude that the structure of the plain muscle machine is similar to that which occurs in skeletal and cardiac muscle, i.e., that each fibril contains minute collapsible sacs, each communicating by a narrow capillary tube with a porous spongy network (doubly refracting towards polarized light), and that changes in the length of the fibre are due to changes in the interfacial tension between the walls of these structures and their fluid content. A diagram of this hypothetical myofibril in the relaxed and contracted conditions is shown in Figure 1. That plain muscle cannot differ essentially from "striated" muscle in its ultimate organization is further indicated by the report of E. J. Carey, who has successfully transformed the plain muscle of the urinary bladder of the dog into rhythmically contracting muscle of the cardiac type (8).

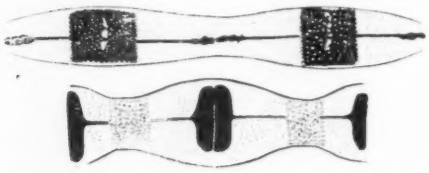

Fig. 1. Diagram to represent the hypothetical structure of two myofibrils, one in the relaxed and the other in the contracted condition.

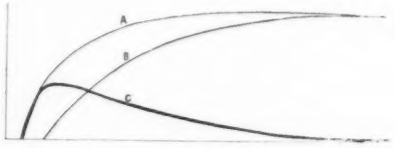

Fig. 2. Curves to illustrate the rate of diffusion of the Acid (A) and Basic (B) substances. By subtracting the ordinates of curve (B) from those of curve (A) the curve (C) is obtained, which represents the quantity of acid at any moment in contact with the walls of the fibrils. Time in abscissae; extent of diffusion in ordinates.*

Each fibril runs throughout the length of the muscle fibre, the nucleus lying somewhat asymetrically, and not in the centre of the fibre. I have not been able to observe whether they are inserted on the cell membrane at their ends.

The Interfibrillar Material is evidently a colourless fluid, and is entirely

* It should be noted that *simultaneous* diffusion of the acid and base will be slower than when the diffusions occur alone. This will cause a prolongation of the time occupied by the "twitch", but not a change in the type of curve.

devoid of granules. What corresponds to the granular sarcoplasm of skeletal and cardiac muscle is found in the form of a mass of granular material surrounding the nucleus. I have previously pointed out that in skeletal muscle the chemical changes that accompany muscle activity must be regarded as taking place at the expense of the interfibrillar sarcoplasm (28). In plain muscle fibres no such sarcoplasm is present, except in the nuclear region of the fibre, from which one must conclude that it is only in the middle of the fibre that the chemical changes which cause contraction occur. This is in agreement with the fact, also, that plain muscle does not contain the excitable membranes of Krause, which are so characteristic of skeletal and cardiac muscle. Since the function of these two membranes is to transmit the stimulus through the fibre, and thereby to bring about chemical changes on their surfaces, we must conclude that *there is no transmission of excitatory impulses within the fibres of smooth muscle.* The objection might be raised that the general protoplasm of the muscle fibres is irritable. But it seems doubtful whether the capacity for transmitting stimuli rapidly is the property of anything but certain very delicate membranes or fibrillae, just visible under the highest magnification. In "striated" fibres there can be no doubt that it is the two membranes of Krause that transmit the stimulus; in nerve cells it seems, though no proof has yet been given, that the rapid transmission of impulses is the function of the neurofibril. In ciliated cells delicate fibrils connect the basal granules of the cilia with the nucleus; and it is apparently the nucleus which, acting through these fibrils, co-ordinates the action of the cilia. I shall try to show below that the behaviour of plain muscle is in agreement with this view of the non-transmission of stimuli within the individual fibres.

(B) *The Innervation of Plain Muscle Fibres.* In further agreement with the view that it is in the granular mid-region of the plain muscle fibre that the chemical changes which produce contraction take place, is the fact that it is here that the fibre becomes innervated. A number of investigators have examined the nerve terminations in plain muscle fibres, and are mostly agreed that the nerves end in the nuclear mid-region of the fibres. My own observations have been made on the musculature of the frog's stomach, stained intra vitam with methylene blue. It is characteristic of this method of staining that only some of the nerves or nerve endings can be stained in a single preparation. Frequently one finds single nerve cells with dendrites or axons that can be traced for very long distances, and though numerous other such cells must be present, yet none of them take up the stain. In the case of the frog, at any rate, a further characteristic is that, while the nerve end-plates, and a small part of the fibre attached to them, may be stained, the remainder of the fibre is unaffected. Again, in some places nothing but the connective tissue elements and some of the muscle fibres may stain, while in other parts these do not absorb the stain at all, but the nerve endings alone are visible. In such places almost every muscle fibre shows the nerve terminations. From

this the conclusion must be drawn that even in those areas where nerve terminations are not visible they nevertheless do occur. This is further shown by the fact that in these apparently nerve-free regions Auerbach's plexus is as prominent as elsewhere.

The occurrence of an independent excitatory and inhibitory innervation of plain muscle tissues, as seen in the vascular system, the alimentary tract, urinary bladder, retractor penis, and elsewhere, suggests that in analogy with the claw muscle of crabs, each muscle fibre might possess not a single innervation, but a double one. My observations were carried out on the muscles of the frog's stomach, and I find that here, indeed, not one, but two nerve fibres terminate on each muscle cell. The two end-plates are characteristically different. Both lie, so far as I could observe, within the fibre, i.e., hypolemally. One is a large, quite prominent branched structure, spreading over the whole nucleus, and at times, so far as I could see, actually wound round it; the other is a very small slightly branched end-plate, and is not applied to the nucleus, but lies in the granular material close beside it. At times only the large nerve ending is to be seen, at times only the small; less commonly the two together. Nevertheless, bearing in mind the capriciousness of the method of staining, we are, I think, justified in concluding that *every muscle fibre in a plain muscle system has a double innervation*. Indeed, when we consider such experiments as those of Pawlow on the adductor muscle of Anodonta (23), where stimulation of one nerve was able to bring about a powerful prolonged contraction, and stimulation of another nerve total relaxation of this muscle, we could hardly expect anything but a double innervation of the fibres. Nevertheless, even in that case the fibres do not enter the muscle directly, but first pass into a ganglion, so that we could not be justified from this fact alone, in the absence of microscopic examination, in ascribing a double innervation to each fibre.

The general arrangement of the nerves of plain muscle will be considered below.

(C) *The Connective Tissue* elements play a much greater part in plain

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Fig. 3.

(1) Portions of six plain muscle fibres from pregnant uterus of rabbit. The myofibrillae and cell membranes are obvious. Four of the fibres are shown in their nuclear region. (Mag. 1900.)

(2) Seven isolated myofibrils from same, showing faint transverse striation. (Mag. 2300.)

(3) Nerve endings of plain muscle fibres. The actual muscle fibres are not shown, but only their nuclei, and, in some cases, the sarcoplasm. In *b*, *c*, *d*, and *f* the double innervation is seen. In *a* only the large, in *e* only the small nerve end-plate is to be seen. In *d* the two nerve fibres are seen originating from one nerve cell.
Frog's stomach. Methylene blue preparation. (Mag. 770.)

(4) A well-developed nerve cell from the nerve network of longitudinal muscle layer (Stomach of frog). Two axons are visible, but are only shown for part of their length.
Methylene blue preparation. (Mag. 410.)

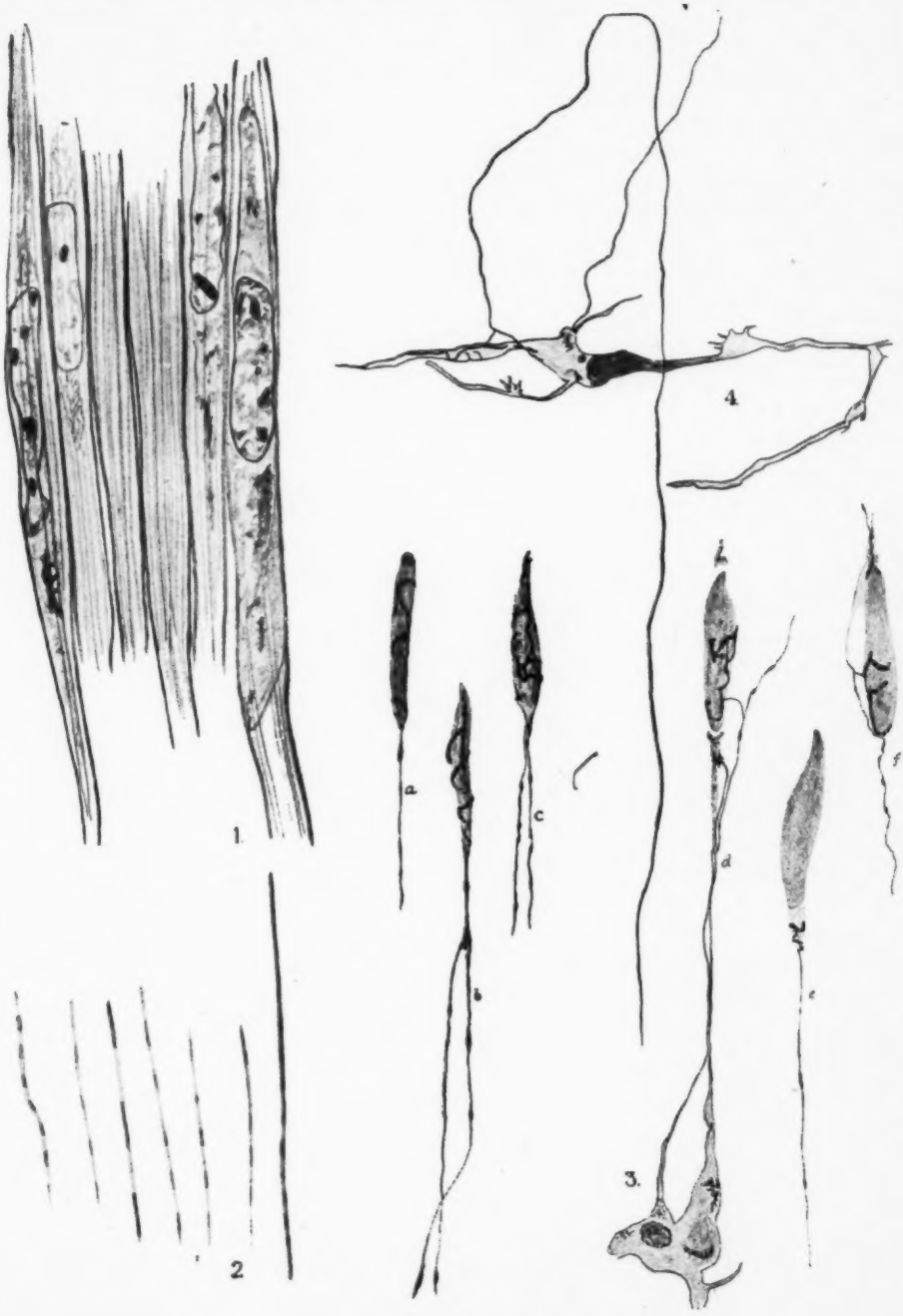

Fig. 3.

muscle than in skeletal. In the latter the main function of the connective tissue is to hold the fibres together, the tension exerted by the fibres being directly applied, at any rate in short muscles, on to the tendons. In large muscles, the interfascicular connective tissue must, however, play a more important rôle. But in plain muscle, where each fibre is but a fifth of a millimeter in length, it is the task of the connective tissue not only to hold these fibres together, but also to transmit the tension which the shortening of the fibre has produced: it plays, indeed, the part of the tendons of skeletal muscle.

Microscopically, the connective tissue is observed, after methylene blue staining, as a thick meshwork holding the fibres together. In the intestinal muscles of the cat it can be seen clearly, and has a very coarse appearance. In the stomach of the frog it is somewhat more delicate. The cells are large, and possess long dendrites, which intertwine with those of neighbouring cells. They stain much darker than do the nervous elements. Various authors—Ramon y Cajal, E. Müller, Schultz (24), Lenhossek, and others—have described them and regarded them as nerve cells. Dogiel (9), however, considers them merely connective tissue cells, and his opinion is, I believe, the correct one. An excellent picture of this network (described, however, as a nerve network by this author) is shown by Schultz in Figure 34 of his paper (24). A characteristic of this connective tissue network, by which it can be distinguished from a true nerve network, which I shall describe below as occurring in plain muscle tissue, is that the processes run irregularly in all directions so as to bind the whole tissue together, and not parallel to the muscle fibres. Occasionally a process may be seen wound round a muscle fibre; it seems generally sufficient, however, that the connective tissue should form a tough network, in which the muscle fibres are firmly held.

The connective tissue meshwork may be compared with a bath sponge. Such a structure is capable of easy compression (with decrease in *volume*, but without change in *form*); of easy stretching (with change of *form*, but no marked change in *volume*). If, now, transverse thickening of a part or the whole of such a structure occurs, it will be accompanied by longitudinal shortening. If, moreover, we imagine the cavities of the meshwork to be filled with minute spindle-shaped elastic bodies, moderately closely packed together, and all arranged longitudinally, then a longitudinal compression of such a sponge-work will result in a proportional thickening of the whole, and this will be accompanied by a sliding of the spindle-shaped bodies past each other, the amount of movement being regulated by the texture of the sponge-work. If, therefore, a transverse section be made through such a sponge in the longitudinally elongated and compressed conditions respectively, then in the latter case more of the spindle-shaped bodies will be cut in a single section than in the former.

Now plain muscular tissue forms such a system. If, then, such a muscle is inserted upon some structure, such as occurs in the various retractor muscles, it can bring about a movement in such a structure. On the other hand, if the contraction takes place in the walls of a flaccid tube, such as the intestine, it can exert a local pressure. The effect of longitudinal compression is well illustrated by the experiments of Grützner (12), and of Müller (21), on the ring musculature of the Amphibian stomach. Grützner finds that while in the distended frog's stomach the walls may be only three relaxed cells in thickness, yet when the stomach is in a state of tonic contraction, during starvation, and when the intragastric pressure is unchanged, the walls may be as much as twenty contracted cells in thickness. A marked "sliding" of the fibres has, therefore, occurred upon one another. Now it will be seen that, as the stomach contracts, the rate of decrease of internal diameter must be considerably greater than the rate of decrease of the outer. Consequently, a compression (which corresponds to the longitudinal pressure in the case of the sponge above considered) will act on the muscle fibres; this will cause a sliding of the fibres upon one another, and this will be most marked in the fibres nearest the mucosa. This "sliding" will, therefore, produce an increase in the area of cross-section of the muscle coat much larger than that actually imposed upon it by the thickening of the cells. Moreover, the rate of increase of the area of cross-section of the muscular coat, as the muscle fibres contract, increases as the *square* of the decrease in circumference, i.e., as the square of the degree of shortening of the individual cells. Now, since the stomach does not decrease in length during this contraction (it becomes, in reality, longer), it follows that general stretching of the connective tissue network must take place; it has already been pointed out that this cannot occur, hence a compensating change must occur, namely, a further decrease in the diameter of the outer circumference, due to a compression of the connective tissue here. This will result in a sliding of the muscle fibres over one another, even in the outer region of the circular layer. It follows, therefore, that during such tonic-contraction the decrease in the outer circumference of the stomach must be greater than the amount of shortening undergone by the fibres in producing it. Experimentally it was observed by Müller (21) that, while the contracted fibres of the stomach are only one-third the length of the relaxed fibres, yet the circumference of the strongly contracted stomach decreases to one-fifth its circumference at relaxation.

The Contraction of Plain Muscle.

(A) *The Simple Contraction Curve.* In recording the twitches of skeletal muscle the damping effect of the "viscosity" of the tissue is largely eliminated by employing maximal stimulation. In plain muscle the inertness of the tough connective tissue meshwork forbids the use of this method; maximal stimulation

of such a tissue would give a fairly accurate representation of the shortening undergone by the fibres, but would give a very false picture of their relaxation. For this reason, too, it would be possible to stimulate a very considerable number of fibres, by what one would falsely call subminimal stimulation, and yet no effect could be detected by a recording lever. Before such a lever could detect any contraction in the tissue as a whole, it would be necessary for a certain minimal number of fibres to have contracted, and to have so increased the rigidity of the whole that the contraction of any other fibres, in excess of this minimal number, would bring about a general shortening of the tissue.

When a contraction curve somewhat in excess of minimal is graphically recorded, it should give a fair representation of the change in length undergone by a single muscle fibre, for the damping effect of the connective tissue meshwork on relaxation will be reduced to a minimum. It should be pointed out that stimulation must be somewhat above minimal, otherwise one would record only the "top" of the contraction curve, the early rise and most of the subsequent relaxation not being detected; it is only then, too, that the time occupied by the shortening and subsequent relaxation could be accurately estimated. Such curves have frequently been obtained by investigators: see Grützner (12), and especially Schultz (25). It is the characteristic of such curves that they rise moderately rapidly, then pass into a brief horizontal, then fall, but not so rapidly as they rose, and gradually sag away. The whole process may last five to six seconds. How are these observations to be interpreted?

The absence of connecting bridges between the fibres shows that there can be no direct conduction of impulses from fibre to fibre (this will be considered more fully below). The absence of the membranes of Krause within the fibres, the accumulation of the sarcoplasm round the nucleus, and the innervation of the fibre in this region, all unite to show that the whole of the chemical changes must occur in a small region at the centre of the fibre near its nucleus, and that the chemical substances, which cause the surface tension change within the fibrils, must diffuse from the middle to the ends of the fibres. Since each fibre measures about ·2-·3 mm. in length, the sluggish contraction curve, as contrasted with the sharp twitch of skeletal muscle, finds an obvious interpretation.

In skeletal muscle shortening is brought about by the liberation of lactic acid on to the walls of the sarcomeres. I have previously tried to show that relaxation from this condition must be due, not to neutralization of the acid by a basic substance constantly present, but by one which is formed simultaneously with the acid. What this base actually is, is not yet known. The fact that in plain muscle the processes of contraction and relaxation can often be dissociated experimentally proves that relaxation is not merely a passing away of contraction, but an active process. Pawlow (23) has already perceived this for the adductor

muscle of Anodonta. The double innervation of plain muscle fibre seems a direct confirmation of this view.

Now, if lactic acid had diffused throughout the interfibrillar space on to the walls of the fibrils, removal of the acid could occur in one of two ways: (a) it might be removed by an increase of the permeability of either the fibrils or the cell walls, allowing the acid to diffuse away, and possibly to become neutralized there by buffers. But the nerve endings do not lie in contact with the fibrils, nor, so far as I can observe, in contact with the cell membrane. Neutralization within the fibrils by a change in the permeability of their walls may be disregarded for reasons to be considered below. Diffusion into the pericellular spaces likewise seems improbable; in skeletal muscle the neutralization certainly occurs in the intrafibrillar spaces, and the fact that plain muscle may be transformed into "striated" (8) would show that there can be no very fundamental difference between the two kinds of fibres. It would, further, be necessary to assume that such an astonishing phenomenon as the oxidative recovery process which the work of A. V. Hill and of Meyerhof has revealed in skeletal muscle (where it must be intercellular) should now occur either in the pericellular spaces, or not at all. The latter alternative would seem very strange, since many of the lower animals employ only plain muscle in locomotion, and yet have a well-developed oxygen-carrying blood system (annulata).

A number of workers, it is true, have noted an apparent increase of permeability of muscle tissues during activity, e.g., McLendon (18). Conductivity experiments were used, but it is clear that increased conductivity might equally well be due to an increase in the electrolyte concentration (especially when an acid is being formed) as to an increased permeability of cell walls. It is, I think, necessary to get away from this idea of changes of permeability of membranes if a clear understanding of muscle action is to be reached.

E. B. Meigs (20) has actually built up a theory of contraction on this idea, analogous to that of McDougall for skeletal muscle. During contraction the permeability of the cell wall is increased, and fluid passes into the pericellular spaces. He finds that the fibres do not thicken (which is false); therefore they must shorten. In confirmation of this, he finds, in sections, an increase in the pericellular fluid. Now, it will at once be seen that if such a tissue shortens, the pericellular fluid seen in any transverse section must automatically increase in quantity. Even if Meigs' view were correct, it might further be asked why after all such a tissue should shorten if fluid left a cell and entered the pericellular spaces?

(b) The only alternative is that the lactic acid is removed within the fibres by direct neutralization, and by a basic substance which is not constantly present, but which is liberated during activity. Plain muscle would then be fundamentally,

at any rate, similar to "striated" muscle. We must assume, in fact, that just as in skeletal muscle an acid is liberated from one excitable membrane and a base from the other, so in plain muscle it is the function of one nerve ending to bring about a formation of lactic acid, of the other to produce the neutralizing base.

Now it is characteristic of plain muscle tissue that it always contains nervous elements which can originate or integrate simple series of impulses. This will be considered more fully below. Cases, such as the apparently denervated intestinal ring musculature of Magnus' experiments or the retractor penis muscle of the dog will be especially discussed. It is here merely necessary to remark that in all cases in which it has been investigated *it is impossible to obtain plain muscle tissue free from an integrating nervous system*, and we are justified in saying that plain muscular tissue is composed of a specialized conducting system, which originates, transmits, or co-ordinates impulses, that the nervous system innervates every muscle fibre of the tissue, regulating the behaviour of the whole contractile system, and that the whole is bound together by a connective tissue meshwork, whose function it is to transmit the tension imposed on it by the contracting muscle cells.

On the assumption that lactic acid and an unknown basic substance are liberated in the nuclear region of the cell, the shape of the contraction curve can readily be explained; it is only necessary to assume that the acid would diffuse faster than the base (which in all probability it would do), and further to assume that the two are not liberated at the same instant (for then they would neutralize each other), but that the base is liberated a little after the acid. It might be the function of the nervous elements to co-ordinate the two actions, or it might merely mean that one chemical action proceeded faster than did the other. If we assume, however, that the two antagonistic substances are really liberated not simultaneously, then it is a simple matter to explain the form of the contraction curve. In the accompanying figure (Figure 2) the curve A represents the extent to which the acid has diffused on the walls of the fibrils, if the acid alone diffused from the mid-region of the muscle fibre. The curve B represents the concentration of the hypothetical base, if this diffused in the absence of acid. From the differences between the ordinates of these two curves we can obtain the curve C, which represents the quantity of acid in contact at any moment with the walls of the fibrils under the conditions under which they appear to occur in plain muscle fibres. The quantity of acid in direct contact with the walls of the fibrils is a measure of the degree of shortening imposed upon the whole fibre, and this is exactly the type of curve obtained experimentally with plain muscle.

(B) *The Nature of Tonus.* The muscle fibres are essentially to be looked upon as under the direct control of the local nervous plexuses, while the nervous system correlates their action with the environment in which they are

placed. This presupposes the existence both of sensory and of motor fibres in these peripheral plexuses. As Sherrington has already pointed out (26), the extraordinary capacity of an organ such as the urinary bladder so to regulate its size that it is able to accommodate widely varying amounts of fluid and yet exert a constant pressure on its contents, is but another example of a reflex postural tonus, for the development of which not only motor, but also afferent, proprioceptive fibres are necessary. The local nervous system may, of course, also be influenced through the central nervous system, as in the vagus and splanchnic control of the intestinal musculature; or at times may be almost entirely under the control of the central nervous system, as when the musculature must react in accordance with the external and not its own environment (e.g., the adductor muscle of Anodonta). In all such cases, however, the musculature can so alter its state of contraction that, regardless of the degree of shortening, it can yet exert a constant tension or pressure on the object opposing it. It seems to me that the explanation of this tonic contraction is that, in response to particular afferent impulses the local nerve plexus reacts in such a way that in many of the muscle cells only the acid-liberating end-plate becomes excited, and the extent to which this takes place will depend on the intensity of the local proprioceptive stimulation. By this means a hollow organ can automatically increase or decrease its size in accordance with its contents. A muscle in an incomplete tonic contraction would, of course, still be able to undergo rhythmic movements.

It will further be seen that a muscle may remain contracted for an indefinite period in this way, and while supporting a heavy weight will be doing no work at all. Experimentally, Parnas observed (22) that the adductor muscle of Anodonta was able to keep the shells closed for long periods, against a heavy weight, and that no increased oxygen consumption could be recorded during this period.

An inhibition of this tonus can immediately be brought about by the stimulation of the base liberating nerves. This might reasonably be expected to be likewise under the influence of local proprioceptive impulses. Experimentally, it has been done by Pawlow for the adductor muscle of Anodonta by stimulating nerves from the cerebral ganglion. When, now, the separate curves given by Pawlow for the tonic contraction and the subsequent active inhibition are examined, both those curves will be found to be identical with the diffusion curves A and B, Figure 2.

In the case then of plain muscle, and also the peculiar crab claw muscle (28), tonus and the inhibition of tonus are merely due to changes of hydrogen ion concentration. It appears to me by no means improbable that the same process may underlie excitation and its inhibition in the central nervous system also.

The explanation which Grützner (12) gives for tonus, namely, that the sliding of the muscle fibres upon each other underlies the phenomenon, is clearly inadequate, and it has been shown above to have a different interpretation.

(C) *The Latent Period of Contraction.* It has been stated (12) that the latent period of contraction diminishes as we increase the strength of stimulation. It should perhaps be pointed out that so far as the individual fibres are concerned there is yet no evidence to prove this. In an excised strip of plain muscle it is, of course, true, but that merely means that in weak stimulation we record only the "top" of the contraction curve, the early part of the "rise" being overshadowed by the inertness of the whole tissue.

The Action of Plain Muscle Systems within the Living Organism.

One of the most widely discussed questions in muscle physiology has been whether the rhythmical movements undergone by plain muscular tissues, as seen, for instance, in the segmentation and pendulum movements of the intestine, have a nervous or muscular origin. That these movements were purely myogenic was stressed by Engelmann many years ago, and received strong support from the experiments of Bayliss and Starling (3), who failed to abolish the rhythmical intestinal movements of the mammalian intestine with nicotine solution of a strength that might be considered sufficient to paralyze all sympathetic ganglia. This observation received further support from the fact that the retractor penis muscle of the dog or cat, which contains no ganglia (10), nevertheless undergoes rhythmical movements when removed from the animal and placed in Ringer's solution. But as a direct contradiction to all these observations came the experiments of Magnus (15), who abolished the rhythmical movements of the circular coat of the intestine by freeing it from all visible traces of Auerbach's plexus. But even such a preparation, apparently quite free from ganglia, could still undergo rhythmical movements, with gradual increase in tonus, under the influence of weak physostigmine solutions (17). Subsequent work by Gunn and Underhill (13), who took the precaution to perform Magnus' operation at low temperatures, so as to avoid shock to the musculature, entirely failed to confirm his early conclusions. Their work is supported by Alvarez and Mahoney (2), and the purely muscular origin of rhythmical intestinal movements is most usually accepted at the present day.

Now, the histological facts above stated prevent us from accepting the idea of myogenic rhythmical movements when applied to plain muscle tissue. It is clearly not legitimate to quote the heart as an analogy; in the heart, connecting bridges occur between adjacent fibres, and the excitable membranes pass along these, as well as along the fibres. We are, therefore, justified in regarding the heart as one enormous muscle fibre. But with plain muscle this is not the case. The case of automatic movements of isolated skeletal muscle in a calcium-free solution has been taken as an analogy (2); but the analogy is clearly false, since

we are concerned, not with the initiation of impulses in a single fibre, but with the co-ordination of these throughout the tissue.

There are, however, two ways in which such impulses might spread throughout the whole tissue:

(a) Possibly the action currents of one fibre might directly stimulate the neighbouring fibres, and if each fibre had a relatively long latent period of stimulation, the slowness of the transmission of stimuli might be explained; one calls to mind the "rheoscopic frog" experiment. Plain muscle is, however, notoriously refractory to electrical stimulation with currents much stronger than those that ever arise in the living cells. It seems, however, possible that this will ultimately prove to be the method by which the embryonic chick heart is excited.

(b) There is the possibility that the tension exerted by a contracting fibre might stimulate others, and so send a contraction wave throughout the whole tissue. But the tension required to stimulate plain muscle is greatly in excess of what the individual cells could ever exert. Moreover, action currents have been described by Alvarez in fatigued plain muscle which was perfectly at rest. We must therefore conclude quite definitely that, just as in skeletal muscle, it is possible to dissociate transmission of excitation and its mechanical effects.

In the earthworm, on the other hand, Biedermann (5) has clearly shown that the peristaltic movements are under the control of the ventral nerve cord. The experiment of Friedländer (11) has shown that such co-ordination need not necessarily take place entirely through the cord, and Biedermann proves that the movements in each segment are really reflex actions incited by afferent impulses from the skin of the segment concerned. Peristaltic rushes appear to be transmitted similarly (1).

How, then, can we explain the rhythmical action of the denervated intestine of Gunn and Underhill's experiment, and the movements of the isolated retractor penis muscle? Now, in the first case, I find that it is impossible to obtain the circular muscle coat of the intestine free from ganglia. I have observed, in sections through the intestine, that ganglia from Auerbach's plexus adhere to the blood vessels as these grow into the muscle ring, and may occur right in the middle of the muscle coat, in small numbers only, it is true, but the preparation cannot be regarded as denervated. I have also seen portion of Peyer's patches drawn right into the muscular coat by the blood vessels. The intestinal musculature is, therefore, not suitable for deciding whether the movements can originate directly in the muscular coat, or whether they have a nervous origin. Other evidences for myogenic rhythmic movements have similarly given way under more careful examination. The ureter, upon whose behaviour Engelmann relied so much, has been shown to contain ganglia; and Brücke (6) finds that the oesophagus of Aplysia is not free from ganglia.

But the retractor penis muscle of the dog, cat, etc., is a muscle which really is quite devoid of ganglia. Fletcher (10) examined it carefully, but could find no evidence of them; I have also examined it, and can confirm his observation. Nevertheless, this muscle, when isolated into Ringer's solution, undergoes very marked rhythmic contractions (30). How are we to reconcile these facts?

The Nerve Network of Plain Muscle. While examining the musculature of the frog's stomach, I observed recently, in the longitudinal layer, a most pronounced nervous plexus, which has not, so far as I am aware, hitherto been described. In intra vitam methylene blue preparations one can see with marked clearness great numbers of cells possessing long dendrites. They communicate frequently with cells in Auerbach's plexus, and axons sometimes of very great length can occasionally be observed (Figures 3 (4). Less commonly the axons are shorter, and cannot be traced any great distance. A characteristic of the axons is that they usually travel between and parallel to the muscle fibres. They are quite different from the cells which Schultz (24) described as nerve cells, the cells there described being in reality connective tissue cells.

Now these nerve cells can be observed, without any difficulty, to communicate with one another: they form, in fact, a nerve network exactly similar to that described by Bethe (4), in the musculature of Medusae. It is a characteristic of this nerve network that synapses, such as occur in ganglia, are quite absent, and the network is able to conduct impulses in all directions. This network is shown in Figure 4 (5).

In the intestine of the rabbit and the cat, I find that a similar network, though of a more delicate texture, is present (Figure 4 (6)). It arises from Auerbach's plexus, and here also the cells anastomose with one another. It is important to notice that this anastomosis of cells is very clear, and that it is on this arrangement that the capacity for conducting impulses in various directions depends. It might be objected that the nuclei which one sees on the fibres are merely neurolemmal nuclei in sympathetic fibres. But the axons of nerve cells in the intestine can be shown to be devoid of such a neurolemma. I find that in the circular layer of the frog's stomach the nerve network assumes the more delicate appearance that it has in the intestinal musculature of the rabbit and the cat.

When we examine the retractor penis muscle of the dog this same anastomosing network can be shown to be present, though larger ganglia are quite absent. Fletcher (10) has already referred to the anastomosis of the nerve cells in this situation.

It seems to me that the discovery of the nerve network* in plain muscle tissue

* The term "nerve network" has frequently been used by authors for the great mass of fine nerve fibres that one often sees in various organs. It should be noted that this is, however, merely a loose name for a mass of nerve fibres, and does not imply an anastomosing system in Bethe's sense of the word.

offers the explanation for the apparently myogenic rhythmical movements. Conduction cannot take place from muscle fibre to muscle fibre, but occurs always through the nerve network. Even when ganglia are present, they cannot act directly upon the musculature, but must always act through this nerve network. It was doubtless this network that was responsible for the rhythmic movements in the "ganglia free" muscle of Gunn and Underhill's experiments, and the physostigmine effect in Magnus' work. We can now also explain the contradictory results of those who have obtained rhythmical movements in "nerve free" preparations, and the observation of Yanase (29) that peristalsis in the foetal rabbit commences on the 26th-27th day, i.e., when the gut becomes innervated. Whether this nerve network is present in all plain muscle tissue I cannot yet say. Dogiel, Bethe, and others have, however, observed such a plexus on the small blood vessels of vertebrates.

When we now examine the behaviour of such a network in the living body the similarity to the movements of medusae are evident. Cannon (7), describing the movements of the ascending and transverse colon, writes: "By making the tonus ring at the caecum, repeated down running waves may be set going; by making a new ring now at the terminus of these waves, reversed waves appear and meet the downward waves progressively nearer the caecum, until only reversed waves are running." He also describes waves running in both directions from a pulsating tonus ring.

The observation of Bayliss and Starling of the relative resistance to nicotine might be quoted as contradicting the above view. It seems to me, however, that no morphological conclusions can be drawn from their work; we can say that the irritable material of plain muscle tissue is more resistant to nicotine than are sympathetic ganglia, but we cannot say that it must therefore lie within the muscle cells. The same criticism might be made to the objection that the intestine can go on beating for days isolated from the body, even after the vascular system has been removed. It should also be noted that a medusa does not possess a blood vascular system.

Now although there can be little doubt that the stimuli for the rythmical movement undergone by isolated ganglion-free strips of plain muscle originate in the nerve network, yet this does not definitely prove that rhythmical movements within the body do not originate from the peripheral ganglia. It seems, indeed,

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Fig. 4.

(5) Nerve network of longitudinal layer of frog's stomach. The large thick "bundles" are parts of Auerbach's plexus. Note the connection of the nerve network with this plexus. Note also the anastomosis of the nerve fibres. Methylene blue preparation. (Mag. 370.)

(6) Nerve network from intestine of rabbit. Note the anastomosis of the fibres and their connection with Auerbach's plexus. Methylene blue preparation. (Mag. 150.)

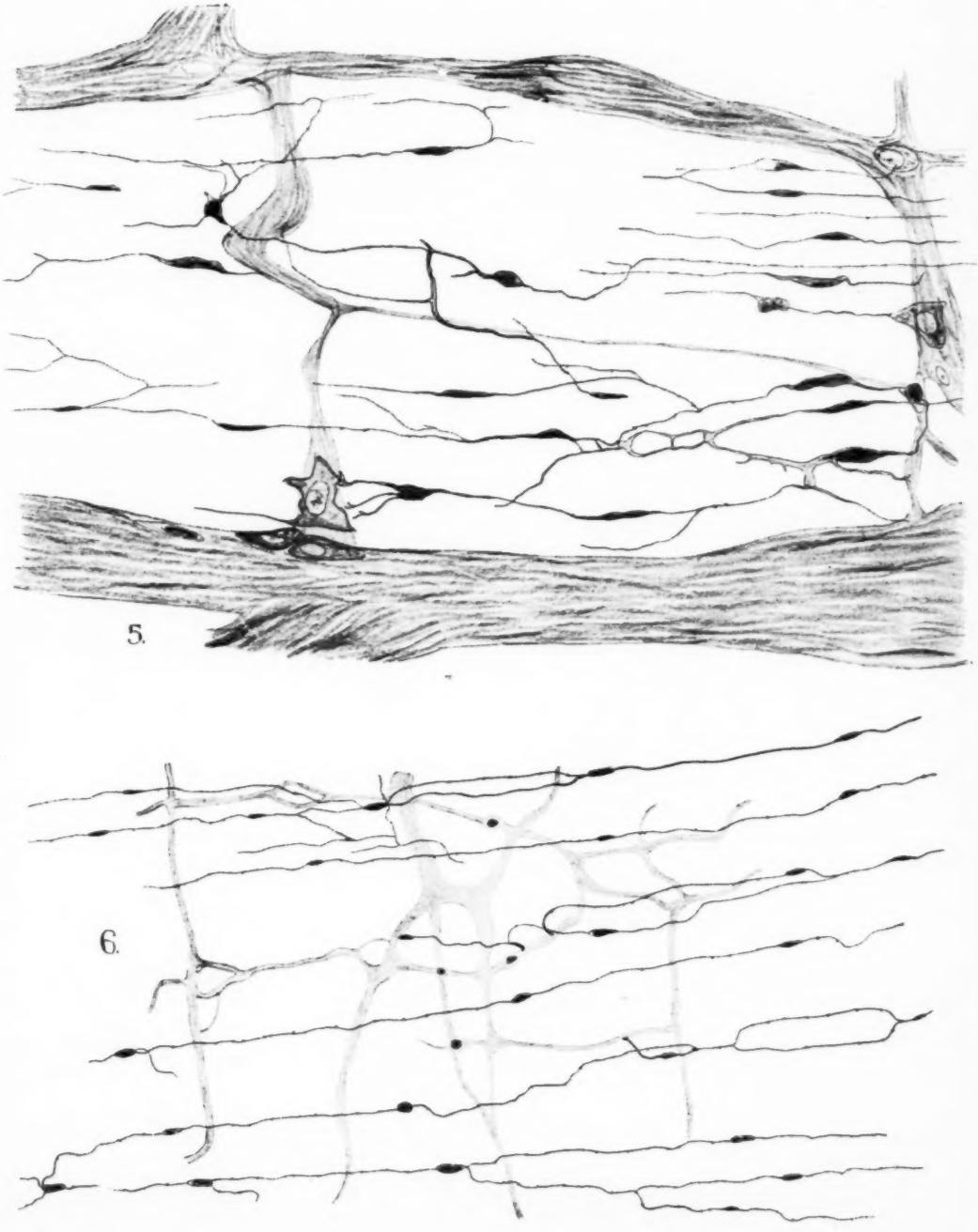

Fig. 4.

probable that the rhythmic movements of these isolated strips are *abnormal* movements: neither the ureter nor the retractor penis muscle shows such movements within the living animal.

There is, on the other hand, I believe, evidence to show that the rhythmical movements undergone by the intestine are really regulated by the local myenteric ganglia. The evidence for this is twofold: (1) It was found by Bayliss and Starling (3) that the two muscle coats of the intestine contract synchronously in the pendulum movements.* It is difficult to see how this synchronous beating could occur, unless there were a common centre controlling its activity. The possible objection based on nicotine poisoning has already been discussed above. (2) It was found by Magnus (16) that if a "ganglia free" strip of circular muscle is stimulated by a tetanizing current it passes into prolonged contraction ("tetanus"). A similar strip containing ganglia responds only by rhythmic movements. Evidently the ganglia exert a profound effect upon the nerve network.

The whole question may best be illustrated by reference to the discussion that has taken place concerning rhythmical movements in medusae. In these animals the ganglia are centralized in a well-defined nerve ring, and these ganglia communicate with a nerve network (of the same type as occurs in mammalian intestinal muscle) which lies in intimate contact with the whole of the musculature. Now it was found by Romanes that the nerve ring portion of the medusa could be separated from the remainder; if this was done then the ganglionated part continued to beat undisturbed, but the ganglion free part remained permanently at rest. This proves that the nerve network does not normally originate the impulses, but merely transmits them from the ganglia. But it was further shown by J. Loeb that if the ganglion free part were now placed in a calcium free solution it would beat rhythmically. Evidently the calcium free solution here exerts an effect on the nerve network which is normally exerted upon it by the ganglia.

Reciprocal Innervation of Plain Muscle Tissue. The common arrangement of plain muscle in places where it surrounds a long tube or a hollow viscus, namely, in a longitudinal and a circular layer, offers a good illustration of antagonistic muscles. Contraction of the circular muscle of a tube can only bring about pronounced elongation if the longitudinal musculature simultaneously

* The actual cause of the swaying can be shown to be due to two causes. Local contraction of part of an intestinal coil, especially of the longitudinal muscle, will tend to alter the centre of gravity of such a coil, and by floating a beating coil in Ringer's solution the effect of gravity in causing the writhing movement can easily be appreciated. The tension exerted by the mesenteries on the intestinal wall tends to prevent compensating movements in adjacent parts of the intestine, and this tends to make the pendular movements more complex still. This can be shown by severing the mesenteries in a coil of beating intestine.

relaxes. We thus see the need for a reciprocal innervation of the two coats, exactly as occurs in antagonistic skeletal muscles.

To what extent this reciprocal innervation occurs in plain muscle systems cannot yet be said. It is conceivable that tonic constriction of a small ring of intestinal musculature might be accompanied merely by a stretching, not relaxation, of the longitudinal musculature. But when contraction of the ring musculature results in the elongation of the tube to thrice its previous length, as occurs, for instance, in the earthworm, then there can be no doubt that here such an innervation really does exist.

If, on the other hand, the two muscle coats contract simultaneously, then the tube will be transformed from a more or less flaccid structure (such as one sees in the earthworm while creeping) into a moderately rigid structure. Now if such a condition be imposed upon an animal such as the earthworm, namely, by the partial tonic contraction of the two muscle coats, then if there occurs a co-ordinated contraction of all the remaining uncontracted longitudinal muscle fibres on one side of the body, the animal will undergo movements quite different from those which it usually undergoes. The latent period of contraction for the whole tissue will be reduced to a minimum, and although the time for each muscle fibre to undergo maximum shortening will be unaltered, yet the co-ordinated partial contraction of numerous fibres in a region where little "slack" need be taken up will result in comparatively rapid movement. This is, I believe, the basis of those rapid writhing movements or sharp avoidance actions one often sees in earthworms. They can be specially well seen if some irritant be applied to the head. If such a wriggling animal be held in the hand, the relative rigidity of the body, in contrast to its normal flaccidity, will be very obvious. Now Biedermann has shown (5) that in the earthworm all movement is neurogenic; it is not necessary, therefore, as some authors have done, to regard these rapid movements as due to a special property of plain muscle tissue, but rather that they are the normal outcome of specially co-ordinated nervous impulses.

SUMMARY.

1. The structure of plain muscle tissue is described.

(a) The myofibrillae are the contractile structures within the fibres, acting on the same interfacial tension principle as skeletal and cardiac muscle. The sarcoplasm is represented by the central granular material of the fibres, surrounding the nucleus.

(b) There is no transmission of stimuli within the fibres, or from fibre to fibre; a true nerve network, similar to that occurring in invertebrates, is

described, and diffuse transmission of impulses is ascribed to this network. Each muscle fibre has a double innervation.

(c) The function of the connective tissue meshwork is discussed.

2. Contraction is the result of diffusion of certain substances (lactic acid and an unknown basic substance) from the centre of each muscle fibre on to the myofibrils. In "twitches" the two substances are liberated simultaneously; tonus is the result of the liberation of the acid only; the subsequent liberation of the base inhibits the tonus.

3. The idea of myogenic rhythmical movements of isolated plain muscle is shown to be untenable, and the origin of the stimulation is ascribed to the nerve network. Evidence is given to show that within the organism plain muscle movement probably is initiated by the nerve ganglia.

4. The necessity for the existence of a reciprocal innervation of the two muscular coats that surround a tube or a hollow viscus is pointed out. Usually contraction of one coat is accompanied by relaxation of the other. Rapid writhing movements are ascribed to co-ordinated contraction of the fibres of a musculature, both of whose coats are in a state of partial tonic contraction.

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THE INFLUENCE OF WASHING UPON THE MULTIPLICATION OF ISOLATED INFUSORIA AND UPON ALLELOCATALYTIC EFFECT IN CULTURES INITIALLY CONTAINING TWO INFUSORIA*

by

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1. *The Anticipated Effect of Washing upon the Multiplication of Isolated Infusoria.*

In previous publications (11, 12, 14) the author has shown that when two organisms (infusoria) are freed from adherent parent culture fluid by washing and then introduced together into the same restricted volume of fresh culture medium, the rate of reproduction is not merely double that displayed by a single individual similarly treated, but some multiple in excess of this varying from 2·5 to 10 or more in different experiments. This effect (allelocatalytic effect) was shown not to depend upon conjugation, which, in fact, does not occur under these conditions in the organism employed (*Enchelys*), and it was, in consequence, attributed to the diffusion of an agent from the organisms into the culture medium which accelerates their reproduction. The presence of this accelerative agent in the culture medium was confirmed in a variety of ways, namely: (a) By the accelerative effect of addition to the culture medium of a small proportion of culture fluid which had been previously inhabited by infusoria. (b) By the enhancement of reproductive rate which occurs when the volume of culture fluid is reduced, so that the quantity of accelerative agent which issues into the medium is less and more is retained within the cells. (c) By the fall of reproductive rate when one of two recently divided individuals is isolated into fresh culture medium, in comparison with the reproductive rate of the other individual which is left in the original medium.

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The occurrence of allelocatalytic effect has since been observed in *in vitro* cultures of fibroblasts (4) of cancer cells (1), and, possibly, also in the growth of vegetable tissues (6). Cutler and Crump (2), however, while confirming the production of agents capable of accelerating division by infusoria (*Colpidium*), failed to observe allelocatalytic effect or enhancement of reproductive rate by reduction of the volume of the culture medium. Greenleaf (5) similarly failed to observe allelocatalytic effect in cultures of *Paramoecium* and *Pleurotricha*, and Peskett has failed to obtain it in a majority of cultures of yeast cells (8).

In all these experiments which have yielded negative results one important element in the technique which the author employed was overlooked. In all of my experiments which yielded positive results, the infusoria, prior to the introduction into fresh culture medium, were washed twice in relatively large volumes of fresh culture fluid to remove adherent traces of the parent culture liquid. The technique, in detail, was as follows: A few individuals were isolated from the parent culture in about 0·003 cc. of the parent culture liquid, and to this minute drop was added about 0·1 cc. of fresh culture medium. From this drop a single organism was removed in about 0·003 cc. of liquid and introduced into about 0·05 cc. of fresh culture medium. From this the organism was again abstracted in about 0·003 cc. of fluid and introduced into about 0·05 cc. of fresh culture medium, forming the final subculture. This process is laborious and tedious, but it is essential for success. In 1921 I had already observed that unwashed individuals usually failed to exhibit allelocatalytic effect, and had attributed it to the contamination of the organisms by sufficient amounts of accelerative agent derived from the parent culture to obscure the increase due to the presence of a second individual, and after the appearance of Cutler and Crump's article I repeated these observations, comparing the effect of introduction of a second individual in washed and unwashed cultures prepared from the same parent culture (14), and I found, as before, that allelocatalytic effect is frequently absent or replaced by actual retardation in unwashed cultures, while it becomes progressively more manifest the more thoroughly the cultures are washed. An apparent contradiction was afforded by the observation to which attention was drawn in my original publication (11), that infusoria repeatedly washed in distilled water, rendered alkaline by the addition of 2 cc. of $\frac{N}{10}$ Na_2CO_3 per 100 cc., fail to exhibit allelocatalytic effect when they are transferred to fresh hay infusion. It was subsequently found, however, that mere immersion in alkalinized distilled water for a sufficient length of time, without washing by repeated transference to fresh alkalinized distilled water, similarly abolished allelocatalytic effect. But it has also been observed that the mortality in cultures thus prepared is extremely high because the organisms are thereby transferred

from a hypotonic to a relatively hypertonic medium, a procedure which is almost invariably fatal, and, if not fatal, greatly retards reproduction (15). Evidently, therefore, failure to obtain allelocatalytic effect in cultures thus prepared may merely be attributable to the death of one of the two individuals, since a large proportion of singly isolated individuals also die. On the other hand, repeated washing in isotonic hay infusion, as I have stated, merely enhances allelocatalytic effect.

In addition to this source of failure to obtain allelocatalytic effect, Greenleaf deferred comparison of his cultures from one and two individuals respectively until they had attained, or nearly attained, maximal population-density. He invariably observed retardation of multiplicative rate due to the second individual. That this result is a necessary consequence of the autocatalytic theory of cell-multiplication will be shown in the third section of this paper.

In my experiments, outlined above, I employed washed infusoria simply because I anticipated that the presence of accelerative agent derived from the parent culture fluid would mask or confuse the results, and I attributed the positive results obtained in my cultures merely to the removal of adherent parent culture fluid by the successive washings. That this explanation may not suffice to explain the results will be evident from the considerations which follow. It will be shown that we have not merely to consider the accelerative agent which is present in the external adherent parent culture liquid, but also that which is present within the cell itself and the distribution or partition of the accelerative agent between the cell and the medium which surrounds it.

If the multiplication of cells is accompanied by the production of an agent which accelerates multiplication, and if the production of this agent is proportional to the number of cells produced, then the rate of multiplication at any moment in a confined volume of culture medium must be given by:

$$\frac{dx}{dt} = k\ x(A - x)$$

where x is the number of cells at the moment (time = t), k is a constant peculiar to the type of cell under consideration, and A is the maximum population attainable, irrespective of whether this is determined solely by the availability of nutrients, or by the accumulation of retarding products, or by both factors combined (13). The integrated form of this equation is:

$$\log \frac{x}{A - x} = K(t - t_1)$$

where $K = kA$ and t_1 is an arbitrary constant which merely indicates the position on the time axis of the point of inflexion in the curve of growth, when the acceleration of growth with time changes from positive to negative.

The applicability of this equation has been established in cultures of bacteria (7) and infusoria (10), and it has also been found applicable to the growth of a variety of higher organisms (13). In the higher animals, however, the complete growth curve is composed of more than one autocatalytic curve or "growth-cycle," the successive curves being superimposed upon one another.

The effect of such superimposition of successive growth cycles is to render the observed value of x at any moment not identical with the value to which the equation applies, but to this value plus an excess due to the growth attained in the previous cycles. Thus x, the observed value, becomes $x^1 + b$, where b is the amount of growth attained in previous cycles (assumed to have been virtually completed at the moment at which the measurement was taken). The apparent value of the maximal attainable growth, A is similarly $A^1 + b$. The differential equation now becomes:

$$\frac{dx}{dt} = k(x - b)\ (A - b - (x - b))$$

that is:

$$\frac{dx}{dt} = k(x - b)\ (A - x)$$

which, when integrated, becomes:

$$\log \frac{x - b}{A - x} = K(t - t_1)$$

The applicability of this equation, more especially to the growth of certain plants, has been established by several investigators (3, 9), and its interpretation is indicated by the method of derivation. Suppose, however, that b were in any instance positive, how should we interpret this? I am not aware that any instance has been discovered in which b is positive in the growth of higher, that is, multicellular organisms which have not been subjected to mutilation. Such a case probably occurs, however, in regeneration, as of the tadpole's tail (13), and, as I am about to show, in cultures of infusoria or other organisms which arise from individuals which are isolated from thickly-inhabited parent cultures.

If a cell is isolated in association with a "charge" of autocatalyst derived from elsewhere and in excess of that producible in the genesis of the single cell, then the rate of reproduction at any moment will no longer be proportional to x, the number of cells produced, but to $x + b$ where b is the number of cells the production of which would be required to give rise to the initial "charge" of autocatalyst. If the autocatalyst is not itself the retarding product of the "master-reaction" of nuclear synthesis (13), but merely proportional to it,[1]

[1] The product, that is, of a side-reaction which is proportional in extent to the main reaction.

then the velocity of multiplication at any moment will be given by:

$$\frac{dx}{dt} = k(x + b)\ (A - x).$$

If, however, the autocatalyst were itself the retarding product of the reaction, then the final attainable population (or mass of multicellular organism) would be reduced by an amount equal to b, that is, A would assume a new value, A^1 equal to A — b. We shall, for the present, confine our consideration to the first and more probable case.[2]

The integral form of this equation is:

$$\log \frac{x + b}{A - x} = K(t - t_1)$$

in which the constant t_1, it will be remembered, is purely arbitrary and determined by the circumstances of the observation, for example, the moment when

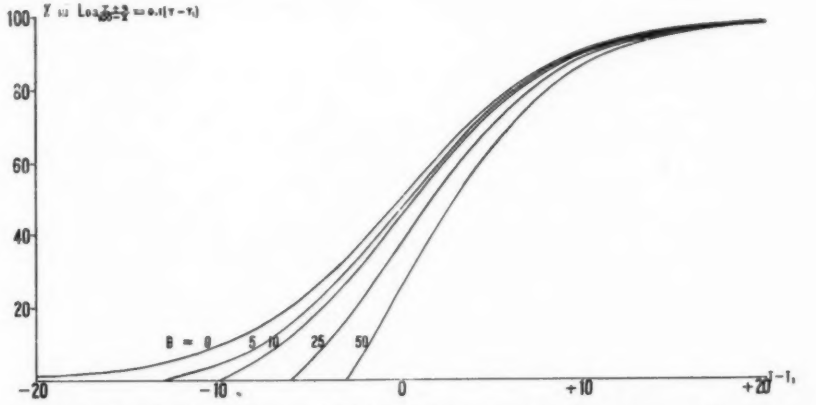

Fig. 1. Showing the effect of initial additions of autocatalyst corresponding to 0, 5, 10, 25 and 50 per cent. of the final mass of the product upon the form of the curve of autocatalysis, the point of inflexion in each curve being fixed at zero time.

fertilization of an ovum occurs, or when a seed is planted, or when an infusorian is isolated into fresh culture medium.

If we give to A the value 100 then x will represent the *percentage* of the final attainable population (or mass of a multicellular organism) which is attained at

[2] Since the most essential product of nuclear synthesis is the chromatin, and it is excessively improbable that this can diffuse from the cell into the surrounding medium while the nuclear membrane remains intact.

any time t. Giving to K the arbitrary value 0·1 and to b the values 50, 25, 10, 5, and 0 per cent. of A respectively, we obtain the series of curves depicted in Fig. 1, the value of t_1 being supposed to be the same in each instance. It will be observed that for all positive values of b certain values of x are *negative*. Such values of x have no physical meaning unless they are regarded as *representing the antecedent history of the organism, prior to the beginning of that multiplication which is actually the subject of investigation.*

Regarding b as a measure of the "charge" of autocatalyst derived from the parent culture (or antecedent growth), and recollecting that at the moment which represents zero time in subcultures of unicellular organisms, the actual population of the culture is the number of individuals introduced into it, assuming that this is one per cent. of the final attainable population, or, which comes to the same thing, that it is unity, and the volume of culture fluid is so chosen as to

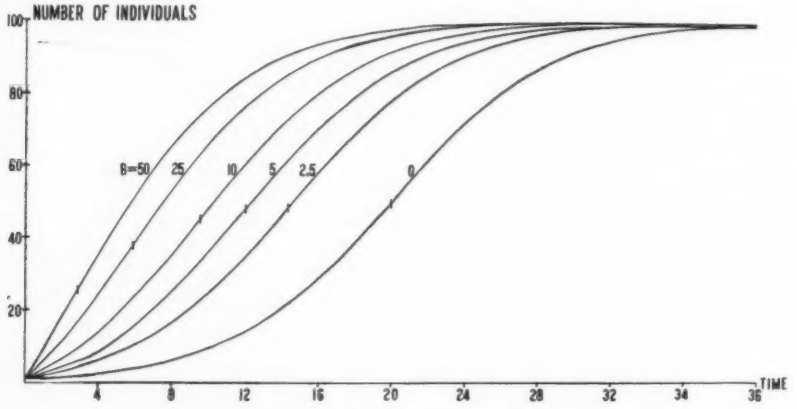

Fig. 2. Derived from Fig. 1 by shifting the curves so that x = 1 per cent. occurs at zero time. This illustrates the effect of initial inclusions of autocatalyst (= B) upon the multiplication of single cells isolated into fresh restricted volumes of culture medium so chosen that the maximum attainable population = 100.

accommodate a maximal population of 100 individuals, then it becomes evident that at zero time the ordinates of the various curves depicted in Fig. 1 will have the same value, namely, 1, and to correspond with the actual conditions of the experiments the curves must be shifted along the time-axis (that is, the arbitrary constant t_1 must be adjusted) until they all coincide at the point $t = 0$, $x = 1$. This done, we obtain the series of curves which is illustrated in Fig. 2. An inspection of these curves reveals the following particulars:

(1) The curves will attain the same maximal value, i.e., if they represent populations of multicellular organisms (or masses of multicellular organisms) then the ultimately attainable population must in every case be the same.

(2) Progressive removal of the initial "charge" of autocatalyst (= b) must lead to progressive diminution of multiplicative rate.

(3) The effect of progressive removal of the initial charge of autocatalyst increases as the "charge" diminishes. This reduction of b from a value corresponding to 50 per cent. of the ultimately attainable population to a value corresponding to 25 per cent. of the ultimately attainable population has much less effect in retarding multiplication than its reduction from 10 per cent. to 2·5 per cent. In other words, if this removal were achieved by successive washings of the isolated organism, then the last washing should be much more effective than the one before, that more effective than the one which preceded it, and so forth.

It will now be shown that the experimental facts coincide with the anticipations outlined above.

2. *The Experimental Effect of Washing upon the Multiplication of Isolated Infusoria.*

For the purpose of these experiments I employed the large species of *Colpidium*, which was used in experiments which have been previously reported (14). This organism is illustrated in Fig. 5A. An average adult individual measures 60 μ by 80 μ. It reproduces by surrounding itself with a gelatinous membrane, within which it divides, usually into four individuals, but sometimes into two.[3] Individuals which are just about to divide are somewhat larger than the average size indicated above, and almost spherical in outline, while individuals which have just issued from the gelatinous membrane are not more than half the normal size.

When maximal population has been attained in any culture, the organisms appear to form the gelatinous covering which usually anticipates division. Instead, however, it shrinks and becomes opaque and tinged with yellow, and the organisms remain encysted indefinitely or until removal to fresh culture medium.

This organism appears very early in cultures formed by steeping various locally obtained plants in tap-water, but it is soon replaced by *Enchelys*, which reproduces much more rapidly, and renders the medium uninhabitable for this species of *Colpidium*. I have repeatedly endeavoured to cultivate the *Colpidium* in association with *Enchelys*, but always with the result that the *Colpidium* died

[3] Especially under circumstances which will be detailed below.

out and the culture became a pure culture of *Enchelys*. The *Colpidium* endures a wider range of P_H than *Enchelys*. Thus I have cultivated it in hay infusion buffered with 1 cc. per 50 of 0·0063 normal acetic acid, containing 0·0603 normal sodium acetate, a mixture which, according to Walpole (16) has a P_H of 5·6. *Enchelys* isolated into hay infusion similarly buffered is killed within an hour.

The culture of *Colpidium* employed contained no other species of infusoria. It contained bacteria which showed a tendency to adhere to one another in clumps. The *Colpidium* was employed in preference to *Enchelys* because its comparatively large size facilitated isolation after successive washings in relatively large volumes of culture fluid.

Unwashed individuals were isolated directly from the parent culture in not more and frequently less than 0·003 cc. of liquid and directly introduced into 0·05 cc. of fresh hay infusion.[4] Washed individuals were introduced with a similar volume of liquid into 0·1 cc. of fresh hay infusion, and from this were re-isolated in a few minutes[5] in about 0·003 cc. of liquid, and either introduced directly into 0·05 of fresh infusion, forming the final culture, or again into 0·1 cc. to be re-isolated before culturing.

The usual effect of washing upon the multiplication of isolated *Colpidia* is illustrated in Figs. 3 and 4, which may serve to represent all of the similar results obtained in a large number of experiments. The multiplicative rate was retarded, and the effect of a third washing was much greater than that of the second (Fig. 4), that of the second greater than that of the first. There was a distinct tendency for the different cultures to attain the same maximal population irrespective of the number of washings, although confirmation of this in the cultures from individuals washed three times was rendered difficult by the extreme slowness of development and the fluctuation of physical conditions (evaporation of culture fluid, etc.), which occurred during the lapse of time necessary to permit attainment of the maximal population. This usually lay between 400 and 500 per 0·05 cc., but in some cultures attained values in excess of 800 (Fig. 4).

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[4] Hay infusion was always prepared in the following manner: Five grammes of chaffed oaten hay were heated to boiling in 100 cc. of distilled water, and the flask containing the infusion was then set upon a boiling water bath for one hour. The mixture was filtered while hot into a steamed flask, and the volume of filtrate made up to 75 cc., if necessary, by the addition of freshly boiled distilled water. The fluid was placed in a 100 cc. graduated cylinder and aerated for one hour by a quick succession of bubbles of air filtered through sterile cotton. To each 25 cc. was then added 1 cc. of $\frac{N}{10}$ $NaHCO_3$ previously saturated with CO_2.

[5] The *duration* of each washing is possibly a factor of importance in determining the magnitude of the result, but as precise quantitative comparisons were not sought no endeavour was made to regulate this factor.

The results are, therefore, such as to indicate that the effect of washing upon the multiplicative rate of isolated infusoria is attributable to progressive removal of an accelerative agent. It at once follows, however, that this agent cannot be

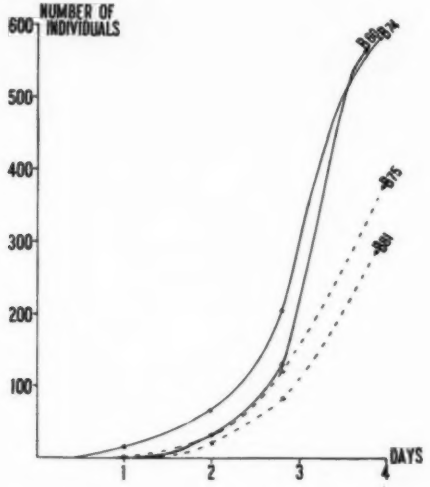

Fig. 3. Showing the usual effect of washing upon the multiplication of isolated infusoria *(Colpidium Sp.)*. The continuous lines represent the multiplication of unwashed individuals, the discontinuous lines, the multiplication of washed individuals. Actual counts indicated by small crosses. B.74 and B.75, individuals isolated from parent culture B.73; B.80 and B.81, individuals isolated from parent culture B.66.

confined to the surrounding medium, but must, to a far greater extent, be contained within the cells. The dilution of the associated parent culture medium in a culture of an unwashed organism is not less than $\frac{6}{100}$, which a single washing must reduce to $\frac{0{\cdot}18.}{100}$ Obviously, further washing could have little or no effect upon the reproductive rate if its modification were solely due to removal of the adherent contaminating fluid. We do not need, however, to suppose that the autocatalyst is actually removed from the nucleus during the process of washing. We may regard the cell and the surrounding medium as comprising three different solvent media, between which the autocatalyst must be distributed

in proportion to its solubility in each, the nucleus, that is, the enveloping cytoplasm and the culture fluid which the organism inhabits. The fact that allelocatalytic effect is displayed only after division has occurred (12) shows that the seat of action of the autocatalyst is the nucleus, and that it is redistributed between the nucleus and its environment (i.e., cytoplasm and culture medium) when the nuclear membrane undergoes disintegration.

If, therefore, the solubility of the autocatalyst is far greater in the cell than in the surrounding medium, then washing may merely serve to remove from the cytoplasm a fraction of its actual inclusion. As the concentration rises in the

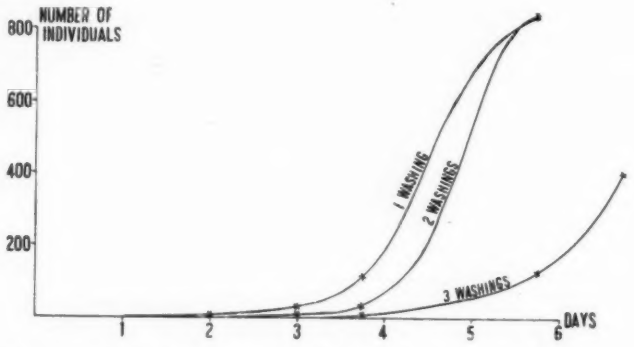

Fig. 4. Cultures B.67, B.68, and B.69, showing the effect of successive washings upon the multiplication of infusoria *(Colpidium Sp.)* isolated from the same parent culture (B.56).

medium with repeated divisions, so, and in the same proportion, it must rise in the cytoplasm and nuclei of the cells, but although doubling of the concentration in the medium must double the concentration in the cell, that concentration may be far greater than the concentration in the medium. The cumulative effect of washing in such relatively large volumes of culture medium as those employed indicates that this is the case, and that only a fraction of the total inclusion is removed at each successive washing. A comparison of Figs. 2 and 4 indicates that even three successive washings, involving a dilution of the associated parent culture fluid to one part in over half a million, may yet not have sufficed to totally remove inclusions of autocatalyst derived from inhabitation of a maximally populated parent culture.

The inclusion of autocatalyst which is still retained after one washing, therefore, may quite possibly correspond to the accumulation resulting from inhabitation of a 50 per cent. maximally populated culture, and thus successive washings would exert the progressively augmenting effect upon reproductive rate

which is actually observed. Very careful quantitative measurements, such as to permit a determination of the actual values of b after successive washings,[6] would, in fact, enable us to ascertain the relative solubility of the accelerative agent in the cell and the culture medium, but such measurements, to yield results of value, would necessitate a considerable number of counts of population at briefer intervals than those employed in the experiments herein described, with more rigorous control of the physical conditions than any yet attempted. The results obtained, however, quite suffice to establish the fact that the greater part of the autocatalyst is contained within the cells which inhabit a culture, only a relatively small fraction of the total issuing into the surrounding medium.

In rare instances the first washing resulted in acceleration of reproductive rate. In every instance in which such acceleration was observed the maximal population was also very appreciably increased. Subsequent washings, however, invariably led to retardation of multiplication. Thus the series of cultures illustrated in part in Fig. 4 yielded the following counts:

Parent Culture B56, maximally populated, seven days old.

Subculture.	Treatment of isolated individual.	Number in subculture after 24 hours.	48 hours.	72 hours.	90 hours.	138 hours.	162 hours.
B66	Unwashed	1	4	16	65	465	—
B67	1 washing	2	8	31	117	834	—
B68	2 washings	1	2	8	32	840	—
B69	3 washings	1	1	2	9	125	403

These occasional instances of acceleration due to the first washing, accompanied also by increase of final population, must have been due, as explained in the first part of this paper, to removal of retarding products as well as autocatalyst. I have never observed any such effect after the first washing, from which it follows that the retarding products are much more readily and completely removed by washing than the autocatalyst. It is, therefore, very improbable that they represent final products of nuclear synthesis, but, rather, as Woodruff has suggested (17), excretory products discharged from the cell into the surrounding medium, and this view is encouraged by that fact that, as shown above, the adherent fluid derived from the parent culture must be almost entirely removed by a single washing.

An effect of washing which was entirely unanticipated, however, was the production of abnormal forms which arose in cultures from highly washed individuals. These abnormal forms are illustrated in Figs. 5, B, C, D, and E. They were usually very small, rapidly rotating ciliated bodies, completely

[6] Subtracting the duration of the period of "lag" (12, 15).

spherical in outline, and devoid of oral depressions, while the normal *Colpidium*, even if small and recently produced, greatly exceeds them in size, is oval in outline, has a deep oral cleft, and swims continually forward with a wavy, swaying movement. Continuous observation of these forms for some hours never revealed any indication of growth, and although the actual death of no one of them was ever actually observed, my observations suggest that they never develop further, but gradually lose motility and ultimately die. Fig. 5D may represent a late stage in this process. The mode of production of these forms is indicated in Fig. 5F. They arise by assymetric division of the parent cell, whereby it divides into a larger part of nearly normal size and one of these relatively minute bodies. This phenomenon was never observed except in cultures derived from repeatedly washed individuals. It was observed in every culture arising from individuals washed three times, only occasionally in cultures arising from individuals which had been washed only twice. It did not appear at once, but after two or three days, when multiplication rate still lagged far behind the normal (Fig. 4). As multiplication proceeded still further they disappeared. Thus in culture B69 (see above) they were seen at 90 and 138 hours, but had completely disappeared at 162 hours, when the culture was rapidly increasing in population. They were never numerous, and not more than six or eight have ever been seen in any culture at one time. Their production, however, seemed to be continuous over a considerable period of time.

No definite interpretation of this phenomenon is at present possible, lacking detailed knowledge of the behaviour of the micro-nucleus during the production of these aberrant forms. It is clear, however, that the excessive removal of occluded autocatalyst consequent upon the thorough washing must represent a condition very unusual in the normal life-history of these organisms, and that in the washed cultures the ratio of autocatalyst to nuclear product must initially lie far below that to which the organisms are habituated. The production of these forms by assymmetrical fission may possibly constitute a reaction to this abnormal disproportion. It may represent, in other words, a phenomenon analogous to differentiation (13).

It was furthermore observed that while in cultures arising from unwashed individuals multiplication took place by division of the parent cells into four, in cultures arising from washed individuals an increasing proportion multiplied by division into two, while in cultures arising from individuals which had been washed two or three times this became the exclusive method of reproduction. Again, in this, we may possibly see the result of a tendency for division to occur at lower nucleo-cytoplasmic ratios in the presence of an unusually low proportion of autocatalyst to the accumulated products of nuclear synthesis.

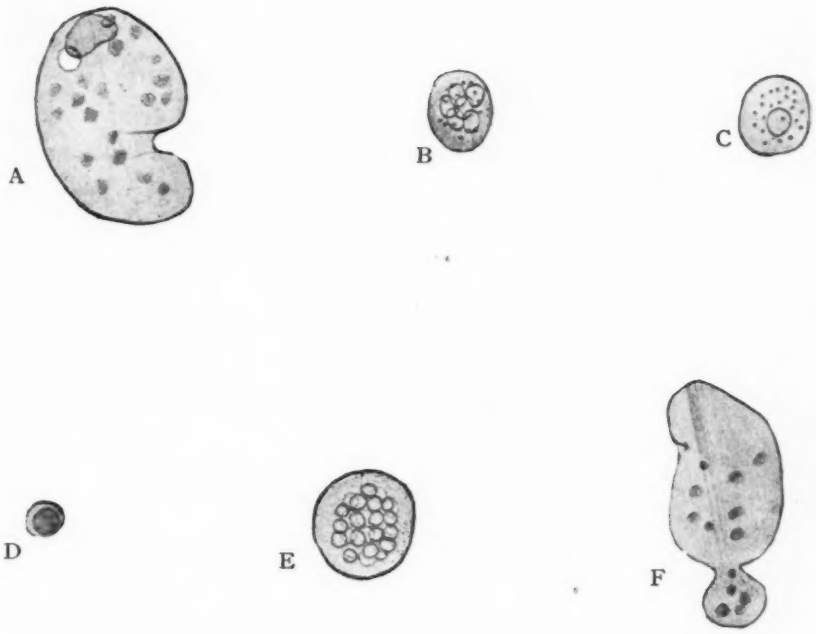

Fig. 5.

A, a normal individual of *Colpidium* Sp. B, C, D, E, and F, abnormal forms found in cultures arising from repeatedly washed individuals. B and C, no mouth, cilia shorter than usual, rotating movement. D, motionless. E, ciliated and undergoing slow rotation. F, swimming rapidly and producing one of the small forms illustrated in B, C, and E by undergoing fission at the posterior extremity. Cilia are not indicated.

(Drawings by Dr. O. W. Tiegs from Culture B.65 on the fifth day. This culture arose from two individuals, each of which had undergone two washings after isolation from the parent culture.)

3. *The Anticipated Effect of Washing upon Allelocatalytic Effect in Cultures of Infusoria.*

If two organisms, each representing a proportion x of the final attainable population A, and each containing an inclusion, b, of preformed autocatalyst, are introduced together into the same restricted volume of culture medium, then, since the maximal attainable population (A) is not affected by the number of individuals originally introduced into the culture,[7] the rate of reproduction at the moment of subculturing is given by:

$$\frac{dx}{dt} = k(2 + 2b)A$$

that is:

$$\frac{dx}{dt} = 2k(1 + b)A$$

which is twice the initial rate of reproduction in cultures inoculated with a single individual.[8] In other words, the effect of the second individual may be considered equivalent to doubling the numerical value of the velocity-constant. We may reach the same conclusion by regarding the phenomenon in the following way. The rate of reproduction of the offspring arising from *each* of the cells introduced into the subculture is represented by the equation:

$$\frac{dx}{dt} = k(x + b)\ (A - x)$$

where x is the *total* number of organisms produced in the culture.[9] Hence the combined rate of reproduction of individuals derived from both sources is given by:

$$\frac{dx}{dt} = 2k(x + b)\ (A - x).$$

In Figs. 6 to 11 the calculated curves of multiplication of single individuals and pairs of individuals in a volume of culture medium arbitrarily chosen to yield a maximal population of 100 are compared, employing the arbitrary value of 0·1 for K in the one case and 0·2 in the other, and recollecting that the initial value of x is, in the one case 1 and in the other 2. The ratio of the ordinates of

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[7] Neglecting of the occasional influence, alluded to in the previous section of this paper, of retarding products contained in adherent parent culture medium.

[8] It must be remembered that A is not diminished by the number of individuals originally introduced, but only by the new individuals which originate in the culture. For this reason the autocatalytic formula, apart from inclusions of autocatalyst, does not strictly apply to the growth of population until x becomes large in comparison with the number of individuals originally inoculated into the culture.

[9] Since each organism produced, whatever its source, contributes equally to increase the concentration of autocatalyst and to diminish the available nutrients.

the two curves, that is, of the numbers of individuals inhabiting the two cultures at one time, is an indication of the degree of the effect of the second individual upon the mean rate of multiplication. If the ratio exceeds 2, it is evident that the introduction of the second individual has resulted in acceleration, while, if it is less than 2, it has resulted in retardation. It is at once evident that allelocatalysis must be almost inappreciable or altogether absent in cases in which the infusoria introduced into the subculture carry with them inclusions of autocatalyst equivalent to that accumulated by inhabitation of a culture containing

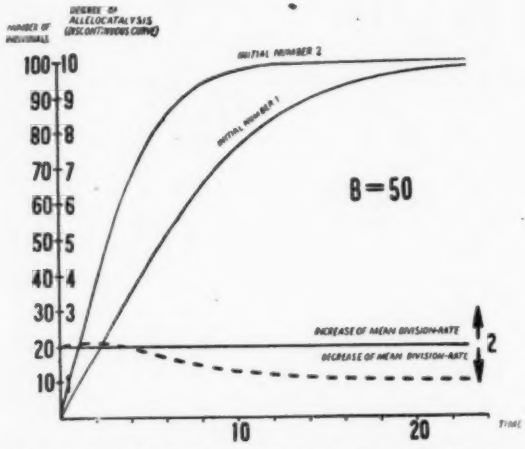

Fig. 6. Showing the effect of an inclusion of autocatalyst corresponding to a population of 50 per cent. of maximum upon the multiplication of a single cell isolated into fresh culture medium and upon the multiplication of two cells introduced together into a similar volume of culture medium. The discontinuous curve represents the ratio of the number of individuals in the culture arising from two to the number in the culture arising from one. Values of this ratio in excess of 2 indicate acceleration of multiplication due to the presence of the second individual (allelocatalysis). Values less than 2 indicate retardation of multiplication due to the presence of the second individual. Volume of culture medium arbitrarily chosen to yield a maximum population of 100.

50 per cent. of a maximal population or more (Fig. 6), and this condition, as we have seen, may readily be realized in cultures arising from unwashed infusoria. This corresponds to the conditions under which Cutler and Crump (2) and Peskett (8) sought and failed to find evidences of allelocatalysis. In the later stages of development in all cases the second individual causes retardation of the mean division-rate, and this corresponds to the retardation actually found by

Greenleaf in cultures arising from two individuals approaching maximal population (5). The conditions observed in my experiments published elsewhere (11, 14) correspond more nearly to those depicted in Figs. 8, 9, or 10.

The reason for the enhancement of allelocatalytic effect by removal of preformed inclusions of autocatalyst is readily seen by reference to Fig. 2. In this the points of inflexion in the various curves are indicated by vertical intersections. It will be seen that the point of inflexion is reached sooner the greater the value of b, that is, the larger the inclusion of preformed autocatalyst. Now, until the point of inflexion is attained, increase of x, that is, increase of the number of individuals, results in increase of the velocity of their production, but beyond

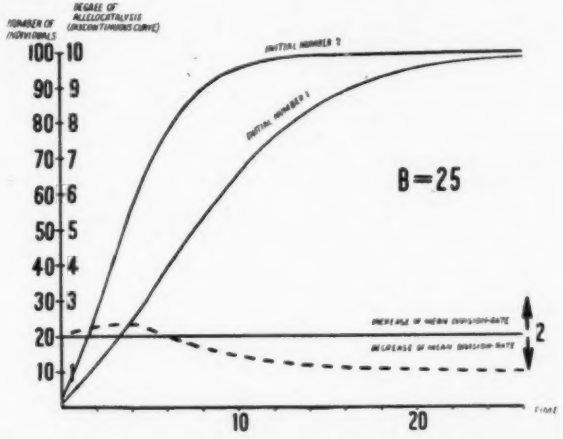

Fig. 7. Showing the effect of an inclusion of autocatalyst corresponding to a population of 25 per cent. of maximum upon the multiplication of a single cell isolated into fresh culture medium and upon the multiplication of two cells introduced together into a similar volume of culture medium. The discontinuous curve represents the ratio of the number of individuals in the culture arising from two to the number in the culture arising from one. Values of this ratio in excess of 2 indicate acceleration of multiplication due to the presence of the second individual (allelocatalysis). Values less than 2 indicate retardation of multiplication due to the presence of the second individual. Volume of culture medium arbitrarily chosen to yield a maximum population of 100.

this point the effect of x is reversed, and that more extensively the further we recede from the point of inflexion. It is obvious, therefore, that as the inclusion of preformed autocatalyst increases, the period during which a second individual can accelerate multiplication is abbreviated and, since the acceleration with increase of x diminishes as we approach nearer to the point of inflexion, the magnitude of the acceleration attainable by the introduction of a second individual into the culture diminishes even more rapidly than its duration.

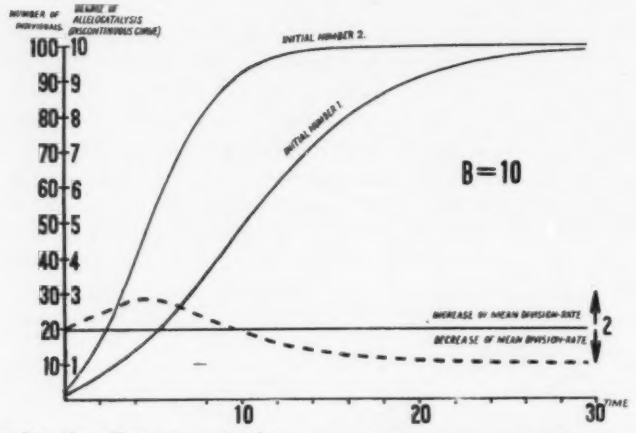

Fig. 8. Showing the effect of an inclusion of autocatalyst corresponding to a population of 10 per cent. of maximum upon the multiplication of a single cell isolated into fresh culture medium and upon the multiplication of two cells introduced together into a similar volume of culture medium. The discontinuous curve represents the ratio of the number of individuals in the culture arising from two to the number in the culture arising from one. Values of this ratio in excess of 2 indicate acceleration of multiplication due to the presence of the second individual (allelocatalysis). Values less than 2 indicate retardation of multiplication due to the presence of the second individual. Volume of culture medium arbitrarily chosen to yield a maximum population of 100.

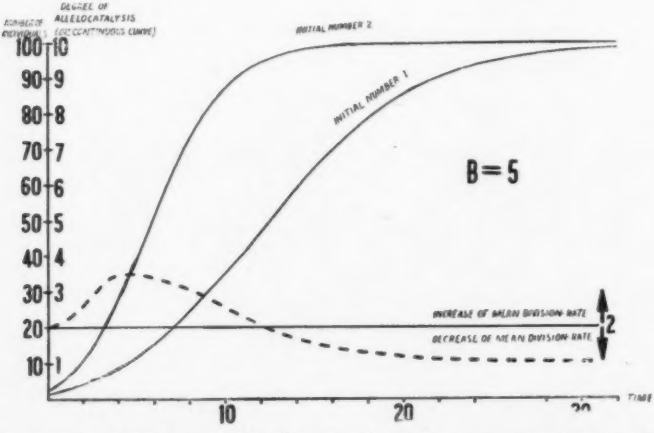

Fig. 9. Showing the effect of an inclusion of autocatalyst corresponding to a population of 5 per cent. of maximum upon the multiplication of a single cell isolated into fresh culture medium and upon the multiplication of two cells introduced together into a similar volume of culture medium. The discontinuous curve represents the ratio of the number of individuals in the culture arising from two to the number in the culture arising from one. Values of this ratio in excess of 2 indicate acceleration of multiplication due to the presence of the second individual (allelocatalysis). Values less than 2 indicate retardation of multiplication due to the presence of the second individual. Volume of culture medium arbitrarily chosen to yield a maximum population of 100.

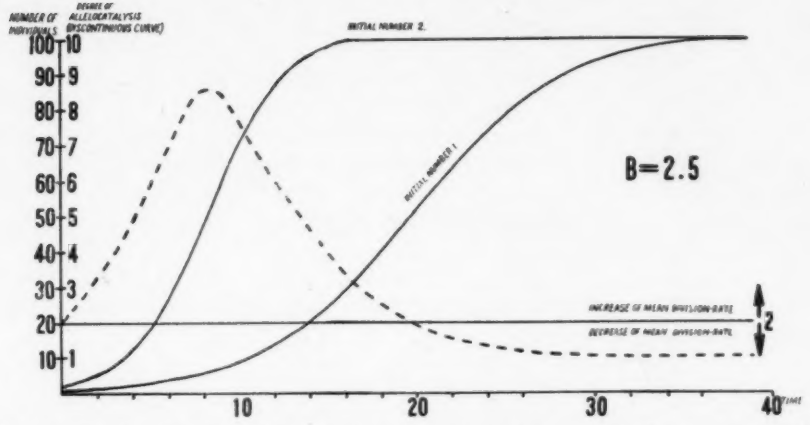

Fig. 10. Showing the effect of an inclusion of an autocatalyst corresponding to a population of 2.5 per cent. of maximum upon the multiplication of a single cell isolated into fresh culture medium and upon the multiplication of two cells introduced together into a similar volume of culture medium. The discontinuous curve represents the ratio of the number of individuals in the culture arising from two to the number in the culture arising from one. Values of this ratio in excess of 2 indicate acceleration of multiplication due to the presence of the second individual (allelocatalysis). Values less than 2 indicate retardation of multiplication due to the presence of the second individual. Volume of culture medium arbitrarily chosen to yield a maximum population of 100.

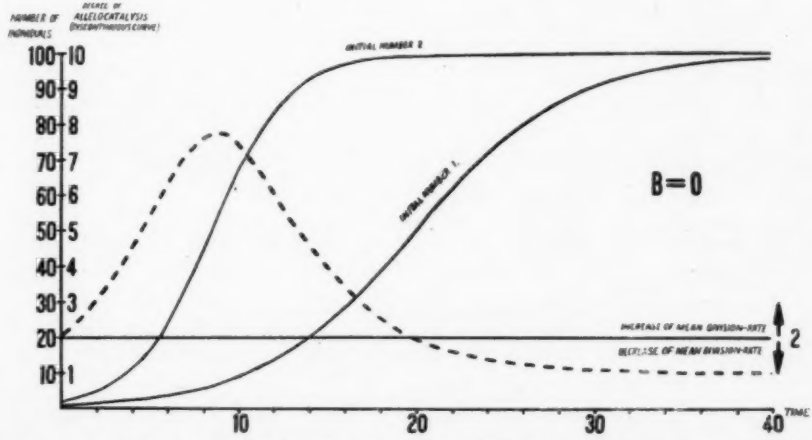

Fig. 11. Comparing the multiplication of a single cell isolated into fresh culture medium with that of two cells introduced together into a similar volume of culture medium in the total absence of any preformed inclusion of autocatalyst. The discontinuous curve represents the ratio of the number of individuals in the culture arising from two to the number in the culture arising from one. Values of this ratio in excess of 2 indicate acceleration of multiplication due to the presence of the second individual (allelocatalysis). Values less than 2 indicate retardation of multiplication due to the presence of the second individual. Volume of culture medium arbitrarily chosen to yield a maximum population of 100.

Inspection of these curves reveals the following additional facts:

(1) Allelocatalytic effect increases with progressive removal of preformed autocatalyst until a maximum effect is attained only slightly prior to its total removal (compare Figs. 10 and 11). The actual magnitude of the maximal effect attainable will depend upon the magnitude of the constant K and the proportion which the number of individuals initially inoculated into the culture bears to the final population.

(2) The effect, when present, increases with lapse of time and growth of population until it attains a maximum value, after which it falls off, at first rapidly and later more slowly.

(3) The ultimate effect of the introduction of the second individual is always retardation of the mean reproductive rate.

It will now be shown that the experimental findings are completely in accord with the effects thus predicted.

4. *Experimental Effect of Washing and of the Age of the Organisms upon Allelocatalytic Effect in Cultures Initially Containing Two Individuals.*

It has already been shown elsewhere (14) that pairs of unwashed individuals of *Enchelys* or *Colpidium*, isolated from densely populated parent cultures, may fail to exhibit allelocatalytic effect, while pairs of sufficiently washed individuals isolated from the same parent cultures exhibit the effect quite distinctly. This aspect of the correspondence between the theory and the experimental findings will therefore not be dwelt upon further. I have, however, made numerous observations upon the increase and decrease of allelocatalytic effect with lapse of time and growth of population. The following are illustrative results:

Parent Cultures five days old. *Enchelys.*

Culture number.	Initial number.	No. after 19 hours.	Ratio of populations.	No. after 43 hours.	Ratio of populations.
310A	1	2	—	16	—
311A	2	3	1·5	120	7·5
310B	1	1	—	4	—
311B	2	4	4·0	57	14·3

Parent Culture three days old. *Enchelys.*

Culture number.	Initial number.	No. after 21 hours.	Ratio of populations.	No. after 22 hours.	Ratio of populations.	No. after 26 hours.	Ratio of populations.
251B	1	4	—	4	—	10	—
252B	2	21	5·2	32	8·0	85	8·5

Parent Culture two days old. *Enchelys.*

Culture number.	Initial number.	No. after 18 hours.	Ratio of populations.	No. after 24 hours.	Ratio of populations.
503A	1	4	—	8	—
504A	2	32	8·0	122	15·3

Parent Culture two days old. *Colpidium.*

Culture number.	Initial number.	No. after 24 hours.	Ratio of populations.	No. after 48 hours.	Ratio of populations.
B15	1	1	—	2	—
B16	2	6	6·0	18	9·0

In all of the above-cited instances the magnitude of allelocatalytic effect increased with increase of population. In those which follow, allelocatalytic effect underwent decrease with increase of population:

Parent Cultures eight days old. *Enchelys.*

Culture number.	Initial number.	No. after 20 hours.	Ratio of populations.	No. after 22 hours.	Ratio of populations.
207A	1	4	—	8	—
208A	2	9	2·25	16	2·00
207B	1	4	—	6	—
208B	2	12	3·00	16	2·67

Parent Culture four days old. *Enchelys.*

Culture number.	Initial number.	No. after 22 hours.	Ratio of populations.	No. after 24 hours.	Ratio of populations.	No after 28 5 hrs.	Ratio of populations.
215B	1	4	—	7	—	16	—
216B	2	14	3·50	20	2·86	36	2·25

Parent Cultures three days old. *Enchelys.*

Culture number.	Initial number.	No. after 21 hours.	Ratio of populations.	No. after 22 hours.	Ratio of populations.	No. after 24 hours.	Ratio of populations.
249B	1	2	—	3	—	4	—
250B	2	6	3·0	8	2·7	8	2·0
255B	1	4	—	—	—	8	—
256B	2	11	2·8	—	—	21	2·6

Parent Cultures three days old. *Enchelys.*

Culture number.	Initial number.	No. after 21 hours.	Ratio of populations.	No. after 22 hours.	Ratio of populations.	No. after 26 hours.	Ratio of populations.
251A	1	5	—	7	—	14	—
252A	2	20	4·0	20	2·8	38	2·7
253A	1	4	—	—	—	9	—
254A	2	9	2·3	—	—	20	2·2
253B	1	4	—	—	—	15	—
254B	2	17	4·2	—	—	51	3·4
255A	1	2	—	—	—	6	—
256A	2	8	4·0	—	—	13	2·2

Parent Culture one day old. *Enchelys.*

Culture number.	Initial number.	No. after 21 hours.	Ratio of populations.	No. after 46 hours.	Ratio of populations.
A128	1	4	—	105	—
A129	2	12	3·0	219	2·1

It is evident from these results that, in accordance with anticipation, the numerical magnitude of allelocatalytic effect, when it occurs, increases at first, and later decreases with increase of population. The fact that all of the cultures finally attain very similar final populations (about 100,000 per cc. in cultures of *Enchelys*) implies that the ultimate effect of the second individual must always be to diminish the mean rate of reproduction (compare Figs. 6 to 11).

As the age and, therefore, the population of the parent culture increases, so also does the inclusion of autocatalyst in each of the cells which inhabit it. It should, therefore, be more difficult to remove the occluded autocatalyst by washing from individuals which are derived from old and densely inhabited cultures than from individuals from young and sparingly inhabited cultures. It follows from this that with a given technique of washing it should be more difficult to elicit allelocatalytic effect, and the effect, when obtained, should be less in extent when the individuals employed are derived from old cultures than when they are derived from young cultures. Numerous observations have been recorded in a previous publication which establish this fact (11), and instances need not, therefore, be multiplied here. Summarizing the results of a number of determinations of allelocatalytic effect, in which the technique employed in washing the organisms was not varied, it was found that the average ratio of the populations after twenty-four hours in cultures originally inoculated with two individuals, compared with those originally inoculated with a single individual from the same parent culture, was 4·6 when the parent cultures were only one day old, and fell to 2·9 when the parent cultures were two to three days old. More effective washing might have yielded higher ratios, but doubtless the values obtained in cultures inoculated from young parent cultures would have remained higher than those obtained in the cultures inoculated from older parent cultures. Still older parent cultures, or less effective washing of the organisms, frequently yield cultures in which allelocatalytic effect is not appreciable, or is replaced at a very early stage in the development of the cultures by a retardation of the mean division-rate due to the initial introduction of the second individual.

A dividing individual would not differ very greatly either in its mass or its inclusion of autocatalyst from any other adult individual inhabiting the same culture at the same moment. Consequently we find that the rate of multiplication of a dividing individual, or of two individuals just formed by fission, does not

differ appreciably from that of any adult individual derived from the same parent culture. The following are illustrative data:

Parent Culture one day old. *Enchelys.*

Subculture.	Population after 24 hours.
55A (Single individual	14
56A (Two individuals)	56
57A (The two fission-products of an individual just divided)	16

Parent Culture one day old. *Enchelys.*

Subculture.	Population after 20 hours.
185A (Single individual)	16
187A (Dividing individual)	17

Parent Cultures, A and B, two days old. *Enchelys.*

Subculture.	6 hours.	22 hours.	24 hours.	28 hours.
234A (Single individual)	1	4	4	8
235A (Dividing individual)	2	5	6	14
234B (Single individual)	1	4	8	14
235B (Dividing individual)	2	6	7	15

The result is very different, however, if the rate of reproduction of a dividing individual, or of the products of its fission, is compared with that of a *single* product of an equally recent fission. The content of occluded autocatalyst must be at a minimum in the product of a recent fission, both because the nuclear mass is at a minimum, and because the recent division has caused a redistribution of autocatalyst between the nucleus and its environment, with release of some of the excess which the parent nucleus had accumulated, in consequence of its own synthesis, during the preceding interdivisional period. Allelocatalytic effect should, therefore, be at a maximum when the organisms employed have arisen from a recent fission.[10] This is, in fact, the case, as the following example shows:

Parent Culture two days old. *Enchelys.*

Subculture.	Population after 18 hours.	Population after 24 hours.
503A (One individual immediately after its production by fission)	4	8
504A (Two individuals immediately after their production by fission)	34	122

[10] Variations of "lag" do not affect the result so long as the single individuals and pairs with which their reproductive rate is compared are of like physiological age, and so exhibit equal durations of "lag." Naturally, however, it is impossible to estimate allelocatalytic effect if one of the pair be derived from an older parent culture than the other, so that its period of "lag" is much longer.

The ratio of populations in these two cultures after twenty-four hours (15·25) approaches the highest numerical value of allelocatalytic effect which I have hitherto succeeded in observing in cultures of *Enchelys*.

CONCLUSIONS.

1. The effects of washing upon the multiplication of infusoria inoculated into fresh culture fluid are chiefly attributable to the partial removal of a "charge" of accelerative agent (autocatalyst of multiplication), which is accumulated by organisms which inhabit crowded cultures.

2. The rate of multiplication is usually diminished by preliminary washing of the isolated organism, and each successive washing induces greater delay of multiplication than the preceding one. The final populations attained, however, are approximately the same.

3. Occasionally the first washing (but no subsequent washing) produces acceleration of reproduction, and in that event the final population is also increased. This effect, when it occurs, is attributed to removal of adherent traces of parent culture fluid.

4. The solubility of the accelerative agent in the cell is much greater than its solubility in the nutrient medium. Hence a single washing in a relatively large volume of the medium removes only a fraction of its total inclusion from the cell.

5. Abnormal forms appear in cultures which arise from repeatedly washed individuals (*Colpidium* Sp.). As multiplication proceeds and becomes more rapid these abnormal forms disappear.

6. Allelocatalytic effect (acceleration of multiplication by the contiguity of a second organism in a restricted volume of nutrient medium) increases with progressive removal of preformed autocatalyst until a maximum effect is attained only slightly prior to its total removal.

7. The effect, when present, increases with lapse of time and growth of population until it attains a maximum value, after which it falls off, at first rapidly and later more slowly.

8. The ultimate effect of the introduction of the second individual is always retardation of the mean reproductive rate.

9. A dividing individual multiplies at about the same rate as any other adult individual derived from the same parent culture.

10. When the reproductive rate of two individuals just produced by fission and inoculated together into fresh culture medium is compared with that of a single such individual isolated into a similar volume of the same medium, the allelocatalytic effect due to the second individual attains its maximal value.

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AN EXPERIMENT ON MUSCULAR RECEPTIVITY

by

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If the facial nerve be cut in an anaesthetized dog, and the peripheral stump brought well out of the wound and stimulated, with due care to prevent spread of current, there will be found in a certain number of cases not only the expected contraction of the muscles involved, but various reflexes showing that afferent impulses, especially of a nociceptive character, have reached the central nervous system. In each of the tracings (Figs. 1, 2, and 3) a decided rise of carotid blood pressure is manifest far greater than could be accounted for by the trifling increase of resistance due to muscular contraction in the facial area; besides there were often reflexes of the nociceptive type involving the skeletal musculature. The animals employed were dogs anaesthized with morphine and chloroform.

In the physiology of half a century ago this effect would be described as "recurrent sensibility," and the explanation would be sought in the suggestion

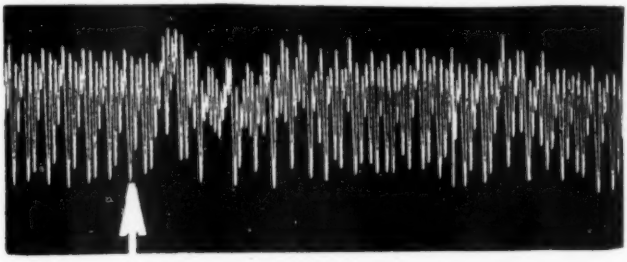

Fig. 1. Effect of Stimulation of Distal End of Facial Nerve.

of an actual confluence of nerve fibres of the seventh cranial with those of the fifth cranial in the peripheral network. According to our present knowledge, this is most unlikely; however, the hypothesis can be easily disproved, for, in

those animals in which the reaction can be repeatedly obtained, light curarization completely abolishes the effect.

There are some curious features about this response. In the first place, it cannot be obtained at all in a large percentage of dogs. When it is elicited, my

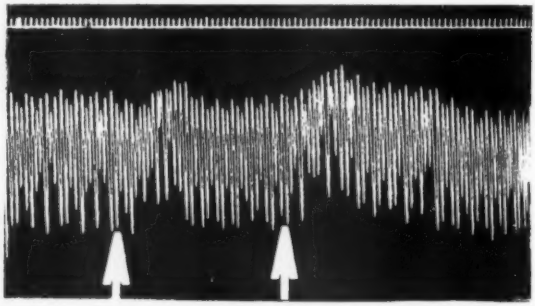

Fig. 2. Effect of Stimulation of Distal End of Facial Nerve. 7 Nov., 1924.

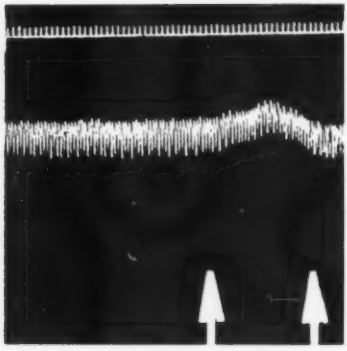

Fig. 3. Effect of Stimulation of Distal End of Facial Nerve. 28 Oct., 1924.

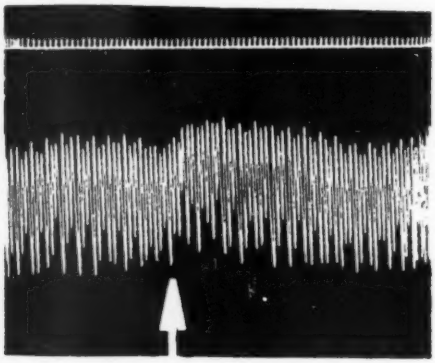

Fig. 4. Effect of Stimulation of Distal Facial after application of Cocaine to the Mucous Membranes.

experience has usually been that it is given once only; repeating the stimulation has produced contraction of the facial muscles, but no evidence of afferent impulses reaching the central nervous system. Even after half an hour's rest the

response does not return. In a few dogs the reflex can be obtained several times, though with diminishing intensity.

It might be urged that the nociceptive impressions arise from unduly stretched mucous membranes. I have disproved this by obtaining the response after cocainization (2% cocaine) of the mucous membranes of the cheek, nostril, and conjunctiva (Fig. 4). It is obvious, therefore, that the impulses must arise from muscle receptors, and it is interesting to observe that they are of the nociceptive type. According to Sherrington (2), "there seems little evidence that active contraction *per se* excites receptors in the muscle itself," and Forbes, Campbell, and Williams (1) also look to "tension as the essential element in the excitation of the intramuscular receptors." In the muscles innervated by the seventh cranial no such tension is developed in contraction as occurs in those muscles that are moving heavy limbs, yet the quick assumption of facial symmetry on cessation of the stimulation shows that there is considerable elastic resistance. It is significant in this connection that I have never been able to obtain the slightest evidence of afferents passing up by any route on the strongest stimulation of the distal end of the cut hypoglossal, though here it is probable that all the fibres from muscle receptors are bound up in the same trunk as the effector fibres.

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THE SEPARATION OF PROTEOSES DERIVED FROM EGG ALBUMIN

by

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Neumeister, Kühne, and Chittenden (11, 8, 1, 2, 3) showed that partial hydrolysis of proteins yielded a number of substances, built up of amino-acids and possessed of distinctly different solubilities in water and salt solutions. Some of these substances were named by Chittenden proteoses. The term proteose is usually employed nowadays to designate those compounds produced by the action of pepsin on proteins, and which are precipitated from solution by ammonium sulphate. Complete investigation of the proteoses has not been carried out, the earlier work on these substances by Pick (12) and by Hart (5) being probably of little value, because complete purification of the fractions obtained was not effected. The most systematic work on the classification of proteoses is that of Haslam (7) on the proteoses contained in Witte's peptone, but other workers, including Levene, van Slyke, and Birchard (9) have prepared isolated proteose fractions from Witte's peptone.

The methods described in this paper are based on Haslam's work, but differ largely in detail. The proteoses formed from egg albumin by the action of pepsin were separated into fractions of different solubilities in water, ammonium sulphate, and alcohol solutions. The scheme of separation was an attempt to realize the following principles, which are necessary if there shall be constancy in the fractions obtained.

1. The concentration of the particular precipitant employed in precipitating a certain fraction shall be such that a definite break occurs in the precipitation, that is, a further small increase in concentration shall not give rise to a further precipitate, but an appreciable increase in concentration shall be required before further precipitation occurs.

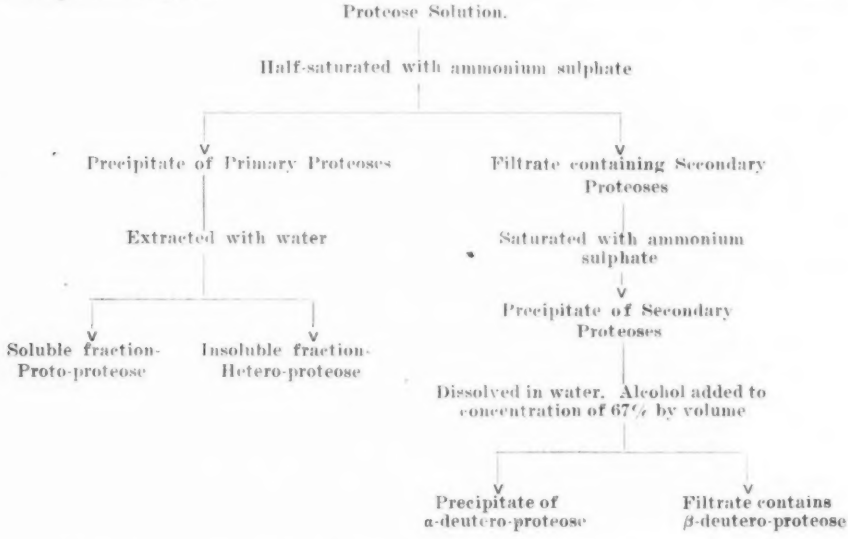
2. When the precipitated fraction is redissolved to the original volume, and the precipitant added until a concentration is reached equal to that employed in the first place to precipitate the fraction, the fraction shall be precipitated

entirely, with the exception of the small amount representing the solubility of the fraction under the particular conditions employed.

3. Similarly, the fractions not precipitated by a particular concentration of precipitant, and which have been first freed from the precipitant, shall remain in solution when the precipitant is again added to its original concentration.

These principles are difficult of realization, a little of the soluble fractions being carried down with the insoluble fractions, and a little of the insoluble fractions remaining in solution, so that the result can be attained only by repeatedly redissolving and reprecipitating. Repeated reprecipitations were carried out in every case until the filtrate from each precipitation showed a constant nitrogen content, which represented the small amount of the precipitated fraction soluble in the particular concentration employed.

The following table gives a brief summary of the methods employed, the nomenclature given by Haslam being followed. It is not suggested that these fractions consist of single substances, but of groups of substances, having similar solubilities in the various solutions employed. Throughout this paper the term "half-saturated ammonium sulphate solution" is employed to mean the solution obtained by the addition of an equal volume of a saturated solution of the salt to an aqueous solution.



Albumin was prepared from hens' eggs by a method similar to that employed by Hartley (6) for the preparation of pure serum albumin. The whites of the

eggs were cut up thoroughly with a knife, and mixed with three times their volume of water. Saturated ammonium sulphate solution was then added in amount equal to the volume of the diluted egg-white, and the mixture allowed to stand overnight. The precipitated globulin was removed by centrifugalization, and the liquid, filtered if necessary, saturated with ammonium sulphate. The albumin was filtered off, dissolved in water, and the volume made up to the original volume of the diluted egg-white. The whole procedure was then repeated, allowance being made for the saturated ammonium sulphate held by the wet, bulky precipitate of albumin, which was estimated approximately by the volume of filtrate obtained in the filtration of the albumin. This process was carried out until the final solution remained clear on half-saturation with ammonium sulphate, indicating the absence of any globulin. The solution was then freed from salt by dialysis in a parchment bag suspended in running tap-water, and finally in distilled water. The albumin was obtained in the solid form by evaporating the solution in a vacuum in a glass jar at 40-45°C., according to the method of Martin (10).

For the preparation of the proteoses a 2% solution of the pure protein was acidified to contain 0·3% hydrochloric acid by weight, and digested with pepsin at 40°C. until the Sörensen titration of the amino-acids became constant, indicating that cleavage of the protein had been carried as far as possible with pepsin-hydrochloric acid. The solution was neutralized with ammonia, and finally saturated with ammonium sulphate. It was then allowed to stand for a day to complete the precipitation of the proteoses. After filtration of the solution, attempted re-solution of the precipitated proteoses in water showed that, whilst the greater part readily dissolved, a fraction was definitely insoluble. It was found convenient, however, not to filter off this precipitate at this stage, but to add an equal volume of saturated ammonium sulphate to the whole mixture, and thus precipitate the primary proteoses.

Purification of Hetero-proteoses.

In dealing with the precipitate of primary proteoses it was found that repeated and prolonged extraction with water at 40°C. removed comparatively little soluble proteose from the precipitate, but if water were replaced by 5% acetic acid nearly all the extractable proteoses (i.e., proto-proteoses) were dissolved within an hour, a second extraction yielding only traces of the soluble proteoses. On neutralization of the first extract (to P_H 7·0 electrometrically) with sodium hydroxide the solution remained clear, and therefore contained but little of the hetero-proteoses, which, as will be shown, were insoluble in water and salt solutions. The solution contained the proto-proteoses.

The residue, after extraction (i.e., hetero-proteoses) was given a prolonged extraction with water, the mixture was filtered, and the nitrogen in the filtrate estimated by the Kjeldahl method. No nitrogen was found, and so the insolubility of the hetero-proteoses in water was demonstrated.

A portion of the hetero-proteoses precipitate was extracted with dilute ammonium sulphate (5%) solution, the mixture filtered, and the filtrate tested for proteose-nitrogen. This could only be done after complete removal of the nitrogen present in the ammonium sulphate. This was effected by heating the solution with sodium bicarbonate (1 gm. for every 1 cc. of saturated ammonium sulphate solution) in an evaporating basin on a boiling water-bath until the ammonia was completely removed. A Kjeldahl estimation on the residue showed that it contained no nitrogen, and thus indicated the insolubility of the hetero-proteoses in dilute ammonium sulphate solution.

Fractionation of Proto-proteoses.

The proto-proteoses fraction which had been extracted from the group of primary proteoses by 5% acetic acid was neutralized with sodium hydroxide, as stated above. Although it could be precipitated by half-saturation with ammonium sulphate, it was thought wiser to employ full saturation with ammonium sulphate in order to remove any possibility of the sodium acetate affecting the result. The precipitate was filtered off and freed from other fractions of proteoses by repeated applications of the following procedure:

The precipitate was dissolved as far as possible in water, and small amounts of insoluble proteoses (i.e., hetero-proteoses) were filtered off. The solution was then half-saturated with ammonium sulphate, allowed to stand for twenty-four hours and the precipitate of proto-proteoses filtered off.

For each purification the proto-proteose was made up to the same volume as that employed in the first place. An estimation of proteose nitrogen was made in the successive filtrates, after removal of the ammonium sulphate with sodium bicarbonate, and was conveniently carried out on samples of 30 c.c. The results of the attempted purification are tabulated below:

	Presence of hetero-proteose.	Proteose-nitrogen in 100 c.c. of filtrate.
1st Purification	Small quantity	24 mgm.
2nd „	Trace	13 mgm.
3rd „	None	14 mgm.

Thus, after three applications of the above procedure, it was found, firstly, that the proteoses were completely soluble in water, and, secondly, that the amount of proteoses which remained dissolved when the solution was half-saturated with

ammonium sulphate reached a small and approximately constant value, which represented, therefore, the very slight solubility of the proto-proteoses in half-saturated ammonium sulphate solution. The primary proteoses had thus been separated into two fractions, the one insoluble in water and salt solutions, and the other soluble in water, but precipitated from its aqueous solutions by half-saturation with ammonium sulphate.

Fractionation of the Secondary Proteoses.

The filtrate obtained from the filtration of a solution of proteoses, half-saturated with ammonium sulphate, contained in solution the secondary proteoses which were precipitated when the solution was saturated with the solid salt. After the solution had stood for twenty-four hours, the secondary proteoses were filtered off, dissolved in a known volume of water, half-saturated with ammonium sulphate, and allowed to stand overnight. A small amount of primary proteoses was precipitated and filtered off. This procedure was repeatedly applied to the filtrate, keeping the volume of the solutions constant, until a precipitate of primary proteoses was no longer obtained, when the solution was half-saturated with ammonium sulphate, and also until a constant quantity of proteose-nitrogen was found in the filtrate. The results obtained are tabulated below:

	Precipitate of Primary Proteoses.	Proteose-nitrogen in 100 cc. of filtrate.
1st Purification	Small	29 mgm.
2nd „	Faint cloud	22 mgm.
3rd „	None	7 mgm.
4th „	None	11 mgm.

Thus, after three precipitations, a fraction of secondary proteoses was obtained free from primary proteoses, and free also from peptones originally present with the proteoses, the small amounts of nitrogen in the filtrates representing the small solubility of secondary proteoses in saturated ammonium sulphate solution.

Separation of α-deutero- and β-deutero-proteoses.

When to a solution of secondary proteoses alcohol is added until its percentage is 67 by volume such a stage of separation of proteoses is reached that the amounts of proteoses in the precipitate formed and in solution are approximately equal. Also, if the precipitate is filtered off the alcohol content of the solution can be increased at least 1½% without further precipitation occurring. Therefore a concentration of alcohol of 67% by volume was chosen to separate secondary

proteoses into two fractions, one soluble in alcohol of this strength and the other insoluble. These fractions have been termed respectively β-deutero-proteoses and α-deutero-proteoses (after Haslam).

The separation of α-deutero-proteoses was effected by repeated precipitation with 67% alcohol in a similar manner to that already described for the primary proteoses. In every case the solution of α-deutero-proteoses, before precipitating, was made to the same volume as that of the original solution of secondary proteoses. Nitrogen determinations in successive filtrates gave the following results:

	Nitrogen in 100 c.c. filtrate.
1st Purification	21 mgm.
2nd „	10 mgm.
3rd „	9 mgm.

Thus, with the removal of β-deutero-proteoses, the nitrogen in the filtrates soon reached a constant value indicating the slight solubility of α-deutero-proteoses in alcohol 67% by volume.

After the removal of α-deutero-proteoses from a solution of secondary proteoses by precipitation with alcohol and filtration, the filtrate, whilst consisting mainly of β-deutero-proteoses, still contained traces of α-deutero-proteoses. Therefore the alcohol was removed from the filtrate by evaporation in a strong air current on a boiling water-bath, under such conditions that the temperature of the solution did not rise above 45°C. The small residual solution was diluted to the volume of the original second proteose solution, and alcohol was added until its concentration was 67% by volume. The slight precipitate which appeared was filtered off, and the whole process repeated. No α-deutero-proteoses were now detected in the β-deutero-proteoses.

DISCUSSION OF RESULTS.

The experiments described indicate that proteoses may be subdivided into the following groups and sub-groups:

I. *Primary Proteoses*: Precipitated by half-saturation of their aqueous solutions with ammonium sulphate, and subdivided into:
   1. *Hetero-proteoses*: Insoluble in water.
   2. *Proto-proteoses*: Soluble in water.

II. *Secondary Proteoses*: Precipitated between half and full saturation of their aqueous solutions with ammonium sulphate and subdivided into:
   3. *α-Deutero-proteoses*: Insoluble in alcohol 67% by volume.
   4. *β-Deutero-proteoses*: Soluble in alcohol 67% by volume.

In the technique of this fractionation of the proteoses no fractions are entirely rejected, so that a qualitative examination of the four sub-groups is equivalent to an examination of the original mixed proteoses. Also, if allowance, based on nitrogen estimations, is made for the portion of each sub-group in the rejected filtrates, quantitative analyses of all the sub-groups together give a result equivalent to an analysis of the original mixed proteoses. In view of the fact that the solubilities of the sub-groups differ among themselves so greatly, it is probable that the sub-groups are decidedly more simple than the proteose solution from which they are derived, and therefore the interpretation of the results of quantitative and qualitative analysis of the former will present a simpler problem than the interpretation of the results of analysis of the latter.

The amount of proto-proteoses obtained was less than the yield of any of the other sub-groups, and a further fractionation of proto-proteoses with alcohol was not attempted, although experiments indicated that probably such a fractionation was possible.

It is proposed to continue this work, and to examine more closely the properties of the different fractions.

In conclusion, I wish to thank Associate-Professor W. J. Young, who suggested this work, and under whose guidance it was carried out.

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PROTEOSES, PEPTONES, AND POLYPEPTIDES

by

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The terms, "proteose," "peptone," and "polypeptide," have been used to indicate successively formed degradation products in the hydrolysis of proteins, but no statement of essential difference between these products has been made. In his work on the synthesis of peptides, Fischer produced tripeptides and tetrapeptides of known composition and structure, which were precipitated when their aqueous solutions were saturated with ammonium sulphate. If these peptides were found in the solution obtained by the hydrolysis of a protein with pepsin they would be classed as proteoses. The analysis of proteoses by the nitrogen distribution method of van Slyke (3) shows the presence of amino-acids of each type which is present in the original protein, and indicates that the proteoses consist either of compounds of a complexity approaching that of proteins themselves, or of a mixture of such comparatively simple peptides as have been synthesized by Fischer. The latter view is to be preferred, in view of the fact that proteoses can readily be fractionated in a variety of ways, but further examination of the fractions is necessary before a certain conclusion can be arrived at. This paper reports experimental evidence bearing on the question.

The solution obtained by the hydrolysis of pure egg-albumin with pepsin was neutralized and then extracted with butyl alcohol, according to the method employed by Dakin (1) for extracting the monobasic amino-acids from a protein hydrolysis solution. This extraction gave rise to three fractions:

1st fraction: Extracted from aqueous solution by butyl alcohol and soluble in butyl alcohol.

2nd fraction: Extracted from aqueous solution by butyl alcohol, but insoluble in butyl alcohol.

3rd fraction: Not extracted from aqueous solution by butyl alcohol.

The first and second fractions obtained thus were examined by the usual qualitative tests applied to proteins and by analysis according to the nitrogen distribution method of van Slyke. The third fraction was treated for the separation of proteoses according to the method described by the author in the preceding paper.

EXPERIMENTAL.

A solution of pure albumin from hens' eggs hydrolyzed with pepsin-hydrochloric acid was prepared according to the method described in the previous paper. After neutralization of the solution with sodium hydroxide, it was extracted with butyl alcohol in a continuous extractor. The butyl alcohol in the distilling flask, in addition to assuming a steadily increasing brown colour due to dissolved peptides, was also seen to contain a brownish precipitate in increasing quantity. The extraction was continued over a period of twenty-four hours, after which time no appreciable quantities of peptides were being extracted. The butyl alcohol was then allowed to cool, and was decanted from the solid peptides in the flask, which was rinsed several times with butyl alcohol, the washings being decanted off and added to the main bulk of the solution. The peptides remaining in the flask were dried in vacuo, whilst the peptides in solution in the butyl alcohol were obtained in the solid state by evaporating the alcohol in vacuo at 40-45° C. Finally, the two dried products were very finely ground and kept in a vacuum desiccator. The yield of peptides soluble in butyl alcohol was approximately 0·2 gm. from 1 gm. albumin, and of the peptides insoluble 0·07 gm. for 1 gm. albumin.

(The peptides insoluble in butyl alcohol were contaminated with sodium chloride formed by the neutralization with sodium hydroxide of the hydrochloric acid originally present in the hydrolyzed solution of albumin. The exact estimation of the sodium chloride was carried out according to the distillation method of Plimmer (1904), which gave the sodium chloride content as 54·9%.)

The results of qualitative tests applied to the two fractions of peptides are given in the table.

Test.	Peptides soluble in Butyl Alcohol.	Peptides insoluble in Butyl Alcohol.
Half-saturation with ammonium sulphate	Precipitate	Precipitate
Mercuric Chloride. . .	Precipitate	Precipitate
Million's Test	Red colour through solution on warming	Red colour through solution on warming
Alkaline lead acetate	No lead sulphide	Lead sulphide appears
Molisch's Test	Doubtful	Positive
Biuret Test	Pinky violet	Pink
Alcohol to 67%	Precipitate	No precipitate

The quantitative analysis of the two fractions of peptides, according to the nitrogen distribution method of van Slyke (3), was carried out in the usual manner. Twenty per cent. hydrochloric acid was used to effect complete hydrolysis, and the end of the action was tested for by determination of amino-nitrogen

in 1 c.c. portions of the hydrolysis mixture. The decomposition of the phosphotungstates of the diamino-acids was effected by the use of a mixture of equal volumes of ether and amyl alcohol, according to a modification proposed by van Slyke (4). The results of the analyses are summarized:

		Peptides soluble in Butyl Alcohol.	Peptides insoluble in Butyl Alcohol.
	Amide Nitrogen . . .	4·48%	7·93%
	Humin Nitrogen . . .	4·09	3·96
	Arginine Nitrogen .	13·74	3·96
	Histidine Nitrogen .	4·54	2·40
	Lysine Nitrogen . . .	3·58	13·20
	Cystine Nitrogen . .	Trace only	Trace only
Filtrate from bases	Amino-nitrogen . . .	65·60	66·65
	Non-amino-nitrogen	2·23	1·62
		98·26%	99·72%

Further analytical results obtained are:

	Peptides soluble in Butyl Alcohol.	Peptides insoluble in Butyl Alcohol.
Total nitrogen	12·09%	12·95%
Amino-nitrogen	3·37	5·76
Amino-nitrogen as percentage of total nitrogen	27·9	44·4

The two fractions of peptides showed marked differences, the most notable being the content in arginine and lysine.

The solution of hydrolyzed albumin after extraction with butyl alcohol was treated according to the method described by the author in the preceding paper, in order to separate the proteoses into the four fractions obtainable by the use of ammonium sulphate and alcohol. Although the fractionation was found to proceed in the usual way, the yields of the hetero-proteoses (primary) and the α-deutero-proteoses (secondary) were very much less than the yields obtained from a solution which had not been first extracted with butyl alcohol.

DISCUSSION OF RESULTS.

The results of the quantitative analyses of the two fractions of peptides isolated by the use of butyl alcohol show that every type of combined nitrogen (with the possible exception of cystine), as estimated by van Slyke's method, is present to a greater or lesser extent. The large percentage of the total nitrogen,

which is present in the form of free amino-groups, is such as would indicate that each fraction consisted of simple peptides. Moreover, the presence of free carboxyl groups was demonstrated by the quantity of alkali required to neutralize the mixture after adding formaldehyde (Sörensen). Therefore each fraction must consist of a mixture of comparatively simple peptides, which differ among themselves in the amino-acid units of which they are constituted. The fact that some, at least, of these peptides are precipitated by ammonium sulphate shows that they belong to the group of proteoses, which therefore are partially, if not entirely, made up of simple peptides. This result is confirmed by the fact that the hydrolyzed albumin solution, after extraction with butyl alcohol, is found to be deficient in both primary hetero-proteoses and secondary α-deutero-proteoses.

It is possible that the terms, "proteose," "peptone," and "polypeptide," will all have to be considered to apply to substances of the nature of peptides, and, possibly, of diketopiperazines, each built up of a small number of amino-acid units and differing from each other, not so much in the number of amino-acids in the molecule, but rather in the variety of these acids. The differences in solubility in various solutions shown by these compounds are thus to be attributed to differences in their chemical constitution, rather than in their molecular complexity. If this be so, these substances would be considered, not as successive stages in hydrolysis, but as products of one completed stage, differing in their solubilities.

In conclusion, I wish to thank Associate-Professor W. J. Young, at whose suggestion this work was begun, for much help and criticism during its progress.

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THE ADJUSTABLE FLEXION TUBE MICROSCOPE

by

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The desirability of having the optical axis of the microscope inclined upwards and backwards, towards the observer, rather than having it in the vertical position, is obvious to every microscopist. Hitherto this has been brought about in ordinary working by inclining the whole tube. This has the following grave disadvantages:

(1) Fluids on the slide or below the slide, such as oil, water, or the like, tend to run off, and are very messy.

(2) Each time the tube is inclined to a different angle the mirror and light require fresh adjustment, and so much time is lost.

(3) Fluids containing material for examination, which are held in watch-glasses or other containers, may be easily spilt, and in any case the surface of the fluid is not at right angles in all its meridians to the optical axis of the inclined tube. In these circumstances, therefore, the tube must be kept vertical, or nearly so, and increased eye, head, and neck strain result.

To provide a tube suitable for general microscopic working I have introduced the flexion tube, as hereinafter described.

1. The device permits of the flexion of the optical axis of the microscope tube in the antero-posterior direction towards the observer, and in the case of the monocular microscope it consists, in one form, of two right-angled prisms placed in the tube of the microscope, one of which prisms (that nearer to the objective, and called herein the "objective prism" and marked "A" in the diagram, Fig. 1) bends the optical axis into the horizontal plane in a direction from side to side relative to the observer, and the other of which (that nearer the ocular, and called herein the "ocular prism" and marked "B" in the diagram, Fig. 1) bends the optical axis again into an antero-posterior vertical plane, but this second prism has, in the moveable type of the device, the power to rotate around a horizontal coronal axis stretching from side to side relative to the observer, so that the optical axis between the ocular prism and the observer may be inclined

in the antero-posterior vertical plane at any desired angle. (See Figs. 2 and 3.) This rotation of the ocular prism around the horizontal coronal axis, so that the reflecting surfaces of the two prisms need not be parallel, is a principal feature of my device. Figure 2 shows the ocular prism ("B") rotated through nearly 45°, so that the light may enter the eye without much bending of the head and without any tilting of the stage of the microscope.

In the fixed type the ocular prism is fixed in a position of partial rotation round the above-described horizontal axis; that is to say, in a position where the

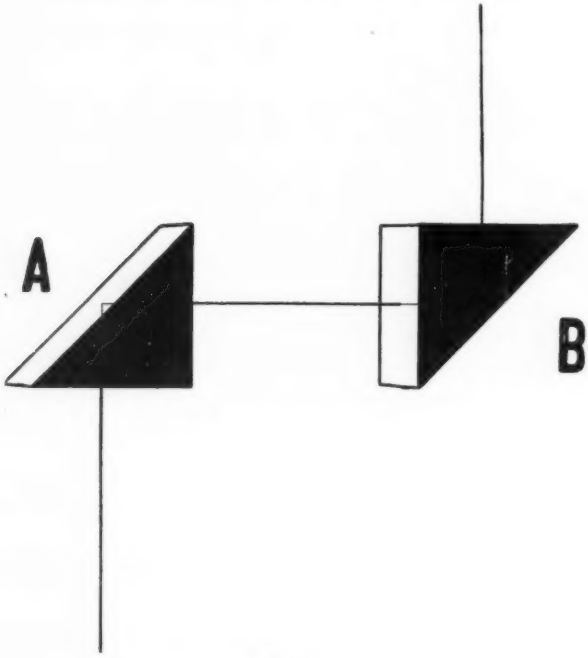

Fig. 1.

reflecting surfaces are not parallel, but at a convenient angle for usual work, so that the advantage of one definite degree of flexion of the tube may be obtained without having the power of varying the angle of flexion characteristic of the complete device. This second arrangement would permit of the production of a cheap type of microscope of fair usefulness.

2. The device may be applied also to the binocular microscope. In this case it may be applied:

A. Before the division of the optical axis has occurred, in which case only one rotating or rotated prism would be necessary. The objective prism would then direct the whole light on to the rotating or rotated prism exactly as in the monocular microscope hereinbefore described, but the rotating or rotated prism would not be near the ocular, for the ordinary prisms used in, e.g., the Leitz parallel tube binocular, first to divide the axis and render the new axis horizontal, and then to bend the new axes into antero-posterior vertical planes, would intervene between the rotating or rotated prism and the oculars.

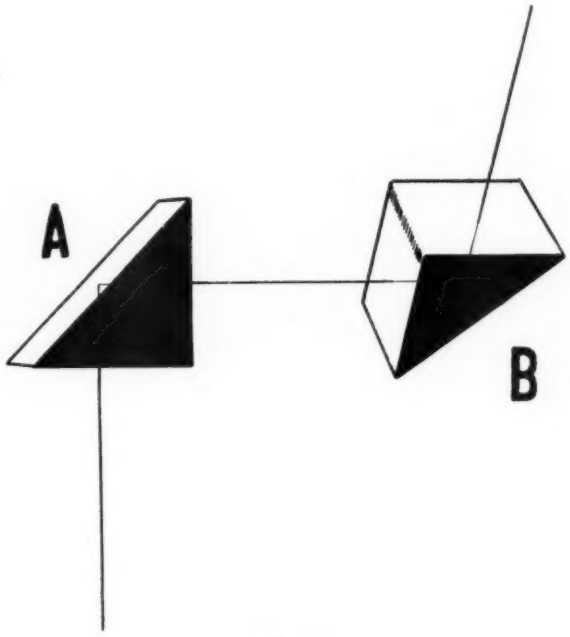

Fig. 2.

B. After the optical axis has been divided, when two rotating or rotated ocular prisms are necessary to enable the optical axes of the two tubes to be flexed in the antero-posterior direction. In the parallel tube binocular microscope at present in use (like that of Leitz, for example) the two right-angled prisms near the oculars have only one movement, that is, the movement permitting of variation of the inter-pupillary distance; their reflecting surfaces are fixed parallel to

the reflecting surfaces of the prisms from which they receive the light, so that the optical axis is directed by them vertically upwards, while in my device the ocular prisms are either:

(i) Capable of rotating round an horizontal axis stretching from side to side of the instrument, so permitting the inclination of the upper sections of

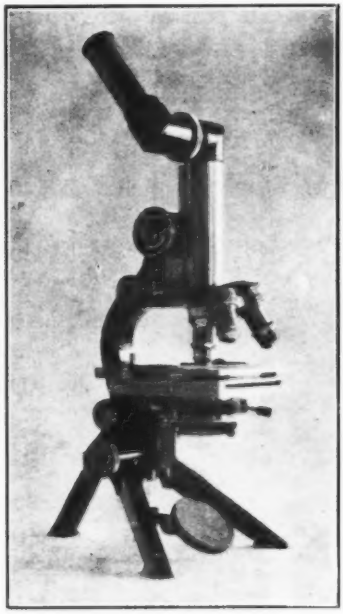

Fig. 3.

the optical axes at any desired angle in the antero-posterior vertical planes; or

(ii) Fixed in a suitable position of partial rotation around the same horizontal axis, so that the upper sections of the optical axes, i.e., those in the parallel tubes, are inclined from the vertical, upwards and backwards, relative to the observer, in two parallel antero-posterior vertical planes, at some arbitrarily chosen useful angle.

In the case B it will be necessary to bring the two horizontal axes into one line. In flexing the tube it is found that the object appears to rotate, and means must be taken in the case of the binocular instrument to make this rotation of the two fields take place in the same direction.

By the use of my flexion tube the upper part of the optical axis, but not the lower, is bent from the vertical, so that disadvantages (1), (2), and (3) above-mentioned can all be obviated, and comfortable working ensured under otherwise difficult circumstances.

Further, floating bodies in the vitreous humour which block the path of light to the macula lutea when the eye is directed vertically downwards may not be exactly in the visual axis when the eye is directed downwards and forwards, or forwards.

The introduction of the flexion device greatly lengthened the ordinary microscope tube to which I first applied it. This was compensated for by the introduction of an achromatic minus lens into the tube below the objective prism.

Practical Experience with the Adjustable Flexion Tube Microscope.

The monocular flexion tube microscope has been in use for bacteriological and clinical pathological purposes for some months now, and has given great satisfaction. It is useful for all types of work where the very highest resolution of the microscope is not necessary, although it seems likely that if the manufacture of the instrument were carried out under ideal conditions, it might very well be used even for work requiring the highest resolution.

For the examination of hanging drops of pathogenic bacteria, blood counts, and the like, it is ideal. It is satisfactory also for the examination of stained films of bacteria, where a magnification of about 700 or 800 diameters is being used.

When the tube is being flexed from the vertical position the field of the microscope rotates, so that if it is used at an angle of about 45° it will be found that the field has rotated a like amount; consequently the markings of the Thoma-Zeiss Haemocytometer will be found to run obliquely across the field, and a little practice is required to compensate for the peculiar movement of the field when the mechanical stage is being used.

The instrument may also be used for making angular measurements of objects in the field, as its angular revolution is exactly proportionate to that of the flexed part of the tube.

It seems likely that the principle of the adjustable flexion tube may be profitably applied to many optical instruments.

My thanks are due to Mr. Goetz for his care in making the first instrument for me, and to Professor Laby, who granted to Mr. Goetz, at my request, the hospitality of his laboratory at the University of Melbourne, where this first sample was made.*

* Patent applied for, 14th August, 1924.